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THE ACTIVITY OF RESPIRATORY ENZYMES AND THE EFFICIENCY OF PROTEIN SYNTHESIS BY SOME AMINOAUTOTROPHIC BACTERIA

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In our preceding communication (Fedorov and Maslennikova, 1960), data were presented on the presence of microorganisms in the composition of the microflora of the gastrointestinal tract of ruminants capable of synthesizing their cell substance from relatively simple carbon (glucose and acetic acid) and nitrogen (urea and ammonium phosphate) compounds. These microorganisms are found in noticeable amounts in the rumen, but gradually decrease in number in the further segments of the gastrointestinal tract. On this basis, it can be assumed that the protein contained in their cell substance is partially utilized by the animal organism. This assumption is confirmed by the data of a number of authors (Popov, 1946; Pearson and Smith, 1943), who showed that the addition of urea or ammonium salts to the animals' rations has a positive effect on them.

We isolated pure cultures of certain representatives of this group of microorganisms and identified them. The following species proved to be the most widely prevalent: 1) Bacillus idosus; 2) Bacterium aerogenes; 3) Escherichia coli citrovorum; 4) Streptococcus salivarius; 5) Pseudomonas fluorescens; 6) Micrococcus radiatus, and 7) Torula (pink and white yeasts). These were the microorganisms which were studied by us in regard to their ability to synthesize cell substance and protein from the nitrogen of ammonium phosphate or urea and the carbon of glucose or acetic acid. Experiments showed that they synthesize protein quite efficiently from such simple compounds. When the amount of protein synthesized by them was expressed in per cent of the weight of carbon source consumed, the data given in Table 1 were obtained. When their supply of molecular oxygen was increased (by shaking or bubbling through the nutrient medium), the productivity of the given process was markedly increased.

From these data it is easily established that the efficiency of protein synthesis depends not only on the properties of the given microorganism, but on the conditions of its cultivation as well. Various carbon sources were used in these experiments, and different ratios of carbon to nitrogen were established in the medium. Both factors had a decided effect on the efficiency of the synthesis of cell substance and the protein which it contained. An even greater effect was found in experiments with increased aeration of the medium, described in the preceding communication. In this connection, it was interesting to determine whether there is a relationship between the activity of respiratory enzymes in the microorganisms under investigation and the efficiency of cell substance and protein synthesis by them. For this purpose, we set up experiments to determine the activity of dehydrogenases and oxidases in the cells of these bacteria.

To determine dehydrogenase activity, we used the Thunberg method, which is based on the determination of the rate of methylene blue reduction by washed

Table 1. The Efficiency of Protein Synthesis by Aminoautotrophic Microorganisms Utilizing the Nitrogen of Urea or Ammonium Salts (average data of two repetitions in % of the weight of carbon consumed)

Microorganisms	Carbon source	Nitrogen source: urea			Nitrogen source: ammonium phosphate		
		Ratio of C : N in medium					
		20 : 1	10 : 1	5 : 1	20 : 1	10 : 1	5 : 1
Bact. aerogenes	glucose	15.20	14.23	10.55	8.27	6.39	5.57
Bac. idosus		10.30	8.00	7.50	11.74	10.03	7.20
Bact. coli citrovorum		29.33	25.6	18.75	12.95	10.37	8.94
Streptococ. salivarius		8.04	7.92	6.33	9.28	8.03	6.74
Ps. fluorescens		11.32	9.24	8.48	7.54	7.71	6.99
Micrococcus radiatus		10.21	7.89	5.89	12.76	11.77	8.70
White yeasts		37.50	36.24	24.51	37.02	34.46	25.13
Pink yeasts		30.11	27.95	26.22	35.48	31.55	27.73
Bact. aerogenes	acetic acid	6.38	6.00	4.40	No growth		
Bact. coli citrovorum		15.96	14.36	11.22	10.43	6.27	5.27
Streptococ. salivarius		11.71	10.54	9.20	14.28	12.08	9.47
Ps. fluorescens		17.23	15.38	11.67	13.68	10.61	8.97
Micrococ. radiatus		9.12	17.90	6.40	14.76	11.82	9.63

Table 2. Dehydrogenase Activity of Certain Species of Aminoautotrophic Bacteria (rate of methylene blue reduction in hours)

Microorganisms	Hydrogen donors and period of decolorization					
	glucose		acetic acid		mannitol	
	start of decolorization	end of decolorization	start of decolorization	end of decolorization	start of decolorization	end of decolorization
Bact. aerogenes	2	3	2	3	3	24
Bact. coli citrovorum	0.25	0.50	0.25	0.50	0.50	1
Ps. fluorescens	1	1.5	2	3	2	6
Micrococ. radiatus	3	4.5	3	5	3	24 (partial)

Table 3. The Intensity of Molecular Oxygen Consumption by Aminoautotrophic Bacteria During the Oxidation of Glucose and Acetic Acid (average data for one hour)

Microorganisms	No. of cells in suspension in millions	No. of cells in suspension in millions					
		O_2 used per μliter, entire suspension			O_2 used per μliter per single cell		
		glucose	acetic acid	mannitol	glucose	acetic acid	mannitol
Bact. aerogenes	3230	57.34	50.85	27.82	$177.4 \cdot 10^{-10}$	$157.5 \cdot 10^{-10}$	$86.3 \cdot 10^{-10}$
Bact. coli citrovorum	3500	68.75	54.61	47.09	$196.7 \cdot 10^{-10}$	$156.2 \cdot 10^{-10}$	$134.6 \cdot 10^{-10}$
Ps. fluorescens	2962	59.15	37.90	24.81	$199.8 \cdot 10^{-10}$	$127.9 \cdot 10^{-10}$	$83.7 \cdot 10^{-10}$
Micrococ. radiatus	3085	49.34	38.21	15.62	$159.8 \cdot 10^{-10}$	$123.8 \cdot 10^{-10}$	$53.6 \cdot 10^{-10}$

cells of the microorganisms under anaerobic conditions. The experiment was set up in Thunberg tubes. Two milliliters of Sorensen's phosphate buffer (pH 6.98) and 2 ml of hydrogen-donor solution in a concentration of 0.05 moles per milliliter of a 1:10,000 methylene blue solution were placed into them. One-milliliter amounts of bacterial suspension containing equal numbers of cells in a given experiment were placed in the side arm. After pumping out the air, the bacterial suspension was poured into the total mixture and observations were made on the rate of methylene blue reduction while the tubes were kept in an incubator at a temperature of 36–38°.

The results of this experiment are given in Table 2.

Glucose proved to be the best hydrogen donor for all of the bacteria tested, but only some of them reduced methylene blue particularly rapidly with the hydrogen mobilized from the glucose being oxidized. E. coli citrovorum was found to be the most active form. It reduced methylene blue completely in half an hour. Methylene blue was also reduced at the same rate by this bacterium due to hydrogen mobilized from acetic acid and mannitol. It had the highest dehydrogenase activity. P. fluorescens was second with regard to the activity of these enzymes, while B. aerogenes was third.

The dehydrogenase activity of these bacteria was also found to be quite high. Complete parallelism could be seen when the efficiency of protein synthesis by these bacteria was compared with these data. These bacteria were also the most efficient in utilizing energy-yielding material for protein synthesis. This pattern is of substantial scientific interest. Therefore, we next studied the activity of the oxidases as well.

To determine the activity of oxidases, we employed the Warburg manometric method, which permits the determination of molecular oxygen consumption by washed bacterial cells.

For these experiments, bacterial cultures were grown on agar slants in large test tubes. Two-day-old cells were washed with phosphate buffer solution (pH 6.98) and were centrifuged at 3500 rpm. After the solution was removed, a thick bacterial suspension was prepared from the sediment, using phosphate buffer solution, and the number of cells in it was counted in a Thom chamber. One-milliliter amounts of this suspension were placed in Warburg vessels to which 1 ml of a 0.1 molar solution of the energy-yielding substance and an additional 1 ml of 0.02 molar phosphate buffer were added. One milliliter of 10% potassium hydroxide solution was placed in the center well to absorb carbon dioxide. Two parallel vessels were set up for each bacterial strain. The experiment was carried on for three hours at 30.3°.

The intensity of molecular oxygen consumption by the different bacterial species also proved to be different. When calculated on the basis of one bacterial cell per hour, the highest indicator was obtained for E. coli citrovorum ($134.6 \cdot 10^{-10}$ μl). Oxidases were found to be the most active in this bacterium. Next to it were B. aerogenes and P. fluorescens. The oxidase activity of these was also quite high. When these data as well are compared with the efficiency of synthesis of cellular material and protein, complete parallelism is again obtained. The higher the oxidase activity of the bacteria tested, the higher is their efficiency of synthesis of cellular material and protein. This regular pattern between respiratory activity and efficiency of protein synthesis is of substantial general biological interest. In actual fact, if a given bacterium has active enzyme systems which oxidize energy-yielding material rapidly, one would expect only its more rapid

growth and more rapid synthesis of cellular material and protein per unit of time. The amount of cellular material and protein synthesized per unit of energy-yielding material utilized, however, would remain a constant value. Nevertheless, our data show that not only does the rate of synthesis of cellular material and protein increase, but the efficiency of the utilization of energy-yielding material for these processes increases as well. Since the processes of synthesis of cellular material are associated with the expenditure of biologically useful energy (the energy of high-energy phosphate bonds), then on the basis of the data given it must be concluded that when the activity of dehydrogenases and oxidases in the cell is high, a large amount of biologically useful energy is released. This, in turn, is possible only when there is a more complete oxidation of energy-yielding material and when there is more complete fixation of the energy released in the course of the oxidative process. Inasmuch as all of the bacteria were cultured under aerobic conditions and the energy-yielding material could have been oxidized to carbon dioxide, it must further be assumed that, with a higher activity of dehydrogenases and oxidases, conditions more favorable for the accumulation of the energy released during oxidation in the form of high-energy phosphate bonds are created in the cell protoplasm. As the result, less energy is disseminated in the form of heat. In this connection, it would be essential to establish at which points of the oxidative process the fixation of energy in the form of these bonds is intensified. Unfortunately, it is impossible to resolve this problem on the basis of the data given. Proceeding from the established fact that oxidase activity is highest in bacteria which synthesize cell material and protein most efficiently, it can only be proposed that the more efficient utilization of oxidation energy is apparently associated with the last steps of the oxidative process, i.e., with the stage where the oxidizable hydrogen is transferred from cytochromes to molecular oxygen activated by the hemin enzyme. As is well known, two high-energy phosphate bonds could be synthesized at this stage of the oxidative process from the energy released (Fedorov, 1957a, 1957b), but only one of them is synthesized in most cases. It is possible that when the activity of dehydrogenases and oxidases is high, an accumulation of energy occurs at this step of the process to the extent of two high-energy phosphate bonds which determine the more economical utilization of energy-yielding material for protein synthesis. However, we still have no direct proof of this proposition, although such a possibility has not been excluded. In view of the fact that this question is of great scientific interest, it should be subjected to further, more detailed investigation.

The data presented show that bacteria isolated from the gastrointestinal tract of ruminants have a highly developed synthetic ability. For example, growing on acetic acid as the sole carbon source and on ammonium salt as the sole nitrogen source, they synthesize all of the substances of which their cells are composed, including complex protein substances, utilizing only the methyl group of the acetic acid for this purpose. If the chemical structure of acetic acid is compared with the chemical structure of protein substances, lipoids, nucleic acids, and other complex organic compounds included in the composition of the bacterial cell, it becomes clear what a variety of the chemical mosaic is produced by these bacteria, starting only from the methyl group of acetic acid, which contains one atom of reduced carbon with three atoms of hydrogen bound to it. For chemical synthesis, such possibilities still remain completely unapproachable. It is therefore entirely natural to suppose that the detailed study of the chemism of biological syntheses in the protoplasm of microbial cells is not only of great scientific interest, but can have practical importance as well, providing materials for the further improvement of the technology of purely chemical syntheses.

In conclusion, it should be noted that the broad study of physiological and biochemical properties in microorganisms encountered in the composition of the microflora of the gastrointestinal tract of ruminants and efficiently synthesizing protein substances while utilizing relatively simple nitrogen-free organic compounds and the nitrogen of urea or ammonium salts will also provide us with material for the understanding of the mechanism of the partial utilization by animals of ammonia or urea nitrogen which is unavailable to them. This nitrogen can be available to them only after the microorganisms growing in the various segments of the gastrointestinal tract of ruminants transform it to the composition of the protein substances of their own cells. After they die off, it is apparently this protein which is partially utilized as a nutrient substance by the animals. Therefore, a more detailed study of these microorganisms is not only of great general scientific interest, but of practical interest as well.

SUMMARY

1. In the gastrointestinal microflora of ruminants great numbers of microorganisms occur which synthesize their cell substance and respective proteins from comparatively simple carbon compounds, such as glucose and acetic acid as well as from urea or ammonia nitrogen.

2. The efficiency of protein synthesis by some of the microorganisms amounts to 29.3–37.5% by weight of the carbon source consumed.

3. A direct relationship exists between the productivity of protein synthesis and the activity of oxidative enzymes (dehydrogenases and oxidases). The higher the activity of these enzymes, the greater the productivity of synthesis of the cellular substance and proteins. Apparently, more favorable conditions for the fixation of energy released in the course of the oxidative process as high-energy phosphate bonds and

the subsequent utilization of this energy for the synthesis of cell material and protein are created when the activity of respiratory enzymes is high. It still remains unclear at which stages of the oxidative process the utilization of oxidative energy increases.

4. The microorganisms we have investigated, which synthesize protein at the expense of the nitrogen of urea and ammonium salt, may be of decided importance in the supplemental feeding of animals with proteins and deserve more detailed study.

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*See English translation.

THE CONSUMPTION BY CHROMATIUM VINOSUM OF ACETIC AND PROPIONIC ACIDS WHEN BOTH ARE PRESENT IN THE MEDIUM

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The clarification of the pathway of carbon in bacterial photosynthesis is of great significance in the understanding of general mechanisms of the autotrophic type of metabolism, and is of particular interest from the evolutionary point of view since this process is related in the evolutionary-genetic respect to other types of synthetic metabolism—heterotrophic photosynthesis, autotrophic and heterotrophic chemosynthesis (Shaposhnikov, 1957).

From this aspect, the study of the character of assimilation of various organic compounds including organic acids is a question of prime importance.

It has been shown in the work of Kondrat'eva (1956) and Nefelova (1955) that cultures of nonsulfur purple bacteria, as well as of typical sulfur bacteria of the genus Chromatium, are capable of growing on mineral medium containing two different organic acids, or an acid and an alcohol. The culture of Chromatium vinosum isolated by us (Osnitskaya, 1958) also grew well on a mixture of two acids judging by the increase in biomass. Subsequently, our work (Shaposhnikov et al., 1960, 1960a) showed that C. vinosum can also grow on certain organic acids—acetic, propionic—added to the medium as the sole carbon source. Work along these lines has been carried out by numerous foreign scientists (Scardovi, 1955; Clayton, 1957; Clayton, Ellingson, and Shaw, 1957; Clayton, Dettmer, and Robertson, 1957; Elsden, 1954, and others) who chiefly used suspensions of nonsulfur purple bacteria in their experiments.

The problem of the present work was the clarification of the dynamics of the utilization of acetic and propionic acids when they were both present in the medium.

The sodium salts of the acids were added in amounts of 0.1% to van Niel's medium without Na_2S and without $NaHCO_3$. In the case where only one acid was added, it was added in the amount of 0.2%. At the end of the experiment, the biomass, cell carbon, amount of acids consumed, CO_2 in the medium, and pH were determined, as well as volatile and nonvolatile acids by the chromatography method. The design of the experiments and the analytical methods were described in the preceding work (Osnitskaya, 1958).

The data of these experiments are given in Tables 1 and 2.

As seen from Table 1, the culture grew well on a mixture of propionic and acetic acids, accumulating as much as 31 mg/100 ml in seven days. In this case, the utilization of both acids, propionic as well as acetic, occurred. During the seven days, ~0.5–1.4 mM of propionic acid was used, and 3–2.6 mM of acetic acid. However, acetic acid was utilized about 1.5–3 times more intensively than propionic, both in millimoles of the acids and when recalculated on the basis of the carbon of each acid. The determination of cell carbon showed that the carbon of acetic and propionic acids was utilized for the synthesis of cell substance. Thus, for example, 11.88 mM of acetic and propionic acid carbon was utilized and cell carbon was equal to 11.88, or 7.78 acid carbon was used, while 7.56 was found in the cells.

It is seen from Table 1 that, as was shown earlier, the culture grew well on each of the acids separately and consumed each of them in larger amounts than when both of them were present: 5.2 mM of acetic acid was consumed in seven days and 2.3 mM of propionic acid.

Table 1. The Growth of Chromatium vinosum on Propionic and Acetic Acids and the Consumption of These Acids in van Niel's Medium without $NaHCO_3$ and without Na_2S

Added to medium	Duration of experiment (days)	pH	Biomass, mg/100 ml	No. of microorganisms, millions/ml	Consumed in mM		CO_2 in cells, mM	CO_2 in medium, mM
					propionic	acetic		
0.1% propionic + + 0.1% acetic	7	7.95	31	38.4	1.3	3	9.9	0.0
0.1% propionic + + 0.1% acetic	7	7.7	23	32	0.43	2.6	6.3	0.0
0.2% propionic	7	7.5	20	27.0	2.3	—	6.8	0.0
0.2% acetic	7	7.6	37	77*	—	5.2	10.5	traces

* Very small cells

Table 2. The Growth of Chromatium vinosum on Propionic and Acetic Acids and the Consumption of These Acids in van Niel's Medium without $NaHCO_3$ and without Na_2S

Added to medium	Duration of experiment (days)	pH	Biomass, mg/100 ml	Consumed in mM		CO_2 in cells, mM	CO_2 in medium, mM
				propionic	acetic		
0.1% propionic + + 0 1% acetic	10	8.0	49	2.1	4.8	15.7	traces
0.1% propionic + + 0.1% acetic	17	8.1	57	2.1	7.5	19	2.5
0.2% propionic	17	7.9	32	3.6	—	10.8	1.0
0.2% acetic	10	7.8	40	—	6.2	12	traces

Table 2, which basically confirms the above-stated, shows that when the duration of the experiment was prolonged (to 10, and even 17 days), the culture continued to utilize both acids and the utilization of acetic acid was more intensive: 7.5 mM of acetic acid (18 by carbon) was consumed in 17 days, while 2.1 mM (or 7.56 by carbon) of propionic acid was used. The carbon of the acid was found chiefly in the cell substance; for example, in 10 days, 19.0 of acids recalculated on the basis of carbon was used, while cell carbon was equal to 18.8.

In experiments lasting 17 days, the evolution of as much as 2.5 mM of CO_2 into the medium was noted, which was most probably connected with the degradation of the culture. No volatile or nonvolatile acids were found in the medium by the chromatography method. In this experiment as well, the consumption of acetic or propionic acids went on more intensively when they were added separately than in the case when both acids were present in the medium.

SUMMARY

1. When grown in a medium containing acetic and propionic acid Chromatium vinosum consumes both acids but the former in greater amounts than the latter (by 1.5–3 times, on the average).

2. The carbon of the acids consumed is found almost entirely in the bacterial cells.

3. In prolonged experiments (up to 17 days) slight concentrations of carbonic acid are found in the medium.

4. As regards the biomass accumulation, the culture grown on both acids exceeds that grown on only one sample.

5. Neither volatile nor nonvolatile acids have been detected chromatographically in the medium except in the acetic and propionic acids which had previously been added to the medium.

6. When both acetic and propionic acids are added to the medium, the consumption of each is less than in a medium containing only one of these acids.

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THE RELATIONSHIP OF BACILLUS MEGATHERIUM TO THE CONDITIONS OF THE MEDIUM IN THE COURSE OF ITS LIFE CYCLE

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The life cycle of spore-forming bacteria begins with the germination of resting spores to physiologically active vegetative cells, and is completed by the transformation of the latter to the resting form once again. As the bacteria pass through the stages of the life cycle, their relationship to the conditions of the medium changes.

It is well known, for example, that the conditions which promote spore formation do not coincide with those required for vegetative reproduction (Ierusalimskii, 1951; Grelet, 1957). Spores are considerably more resistant to unfavorable effects of the medium than are vegetative cells. However, the latter can apparently differ in their resistance as well. In any case, this is true with regard to nonsporogenous bacteria which, in the transition from the resting phase ("lag phase") to the phase of rapid ("logarithmic") reproduction, exhibit increased sensitivity to heating, salts, dyes, and other harmful factors (Dubos, 1948). On the other hand, such young cells develop adaptive enzymes more easily than cells which had gone on to the state of rapid multiplication (Hegarty, 1939).

The process of the conversion of young vegetative cells to physiologically mature ones, and the latter to forespore and spore forms, takes place relatively slowly and gradually. However, the germination of resting spores to young vegetative forms which again start the life cycle denotes a rapidly changing physiology of the cells. It can be assumed that this is accompanied by equally sharp changes in their relationship toward the conditions of the medium.

The enumerated questions have been studied very little as yet. Therefore the further investigation of the relationship of spore-forming bacteria to the conditions of the medium is of decided interest.

METHODS

A culture of Bacillus megatherium served as the experimental material in the present work. The media employed were of the following composition:

1. Synthetic medium (in %): 0.1 NH_4NO_3; 0.05 K_2HPO_4; 0.05 $MgSO_4 \cdot 7H_2O$; 0.025 NaCl; 0.001 $MnCl_2$; 0.0007 $CoNO_3$; 0.25 glucose; 0.2 succinic acid. The medium was neutralized to pH 7.

2. Medium made with meat water: mineral salts and glucose added to meat water in the amounts indicated above; succinic acid omitted.

3. 10% meat water: differs from preceding medium in that it is prepared with meat water diluted 10 times.

4. Meat-peptone medium (MPB) and the same medium in agar form (MPA) – standard composition.

EXPERIMENTAL DATA

The medium made with meat water and MPB, which are rich in carbon and nitrogen nutrients, was more favorable for vegetative growth and reproduction than the other two media. This is seen from the data given in Table 1 on the growth of the B. megatherium culture in flasks on a shaker.

With regard to the process of spore formation, according to available data (Halvorson, 1957; Grelet, 1957), in aerobic ammonifiers this process proceeds better on media which are deficient in nitrogen or carbon nutrients. For a more precise determination of the conditions required for spore formation, the exchange of media method (Ierusalimskii and Rukina, 1956; Ierusalimskii, 1958) was employed in our laboratory. With this, it was established that in certain agents of fermentative processes (Cl. saccharobutyricum, Bacillus macerans), the transition from vegetative reproduction to the formation of spores is associated with the disappearance of nitrogen sources from the medium.

In the present work, instead of exchangeable media, a simpler method using double-layer agar was employed which consists of the following. First, "starva-

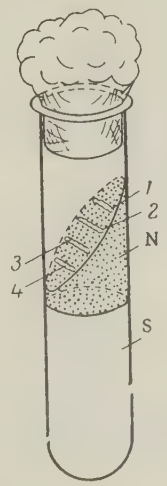

Fig. 1. Diagram of test tube with nutrient agar slant with an under-layer of "starvation" agar. N) Nutrient agar; S) starvation agar; 1, 2, 3, 4) culture inoculated in streaks.

tion" agar prepared with distilled water was poured into Petri dishes or wide test tubes (3–4 cm in diameter), and on top of it—a layer of nutrient agar. In cases where test tubes were used, the agar was slanted and bacteria were inoculated on its surface in transverse streaks (Fig. 1). In the time that it takes for the inoculated culture to grow out into cell aggregates visible with the naked eye, the nutrient substances diffuse into the lower layer while the upper layer becomes impoverished, which promotes spore formation. The composition of the nutrient medium is, of course, very important as well.

In Table 2, data are given on the intensity of spore formation by B. megatherium on various media with and without under layers of starvation agar. A rich medium such as MPA was the least favorable for spore formation. In this case, even sublayering did not lead to mass sporulation, although it increased the percent of sporulating cells considerably. Sporulation proceeded much better on the other two media which were poorer in carbon nutrients. Sublayering of the media with starvation agar intensified this process even more. It should be noted that the bacteria sporulated most intensively in the two bottom streaks of the sublayered agar, where the medium was exhausted of nutrient substances particularly rapidly. On control agar, on the contrary, sporulation was less intensive in these streaks than in the upper ones, because their nutrient reserves were more easily replenished due to nutrient substances diffusing from the underlying layers of agar.

Thus, the media which stimulated sporulation were the least favorable for vegetative growth. This is clearly seen when the data in Tables 1 and 2 are compared.

A complete medium containing all those nutrient elements which are necessary for vegetative reproduction is required for spore germination. This question is quite clear. It is also well known that spores are more resistant to unfavorable effects than vegetative cells; this is precisely the biological significance of sporulation. The question of the comparative resistance of mature vegetative cells and of physiologically young cells which have just formed from spores has been investigated considerably less.

In order to illuminate the given question, we tested the sensitivity of both types of cells to norsulfazol—one of the sulfamide preparations used in chemotherapy.

Microscopic observations of a culture growing on the surface of a thin agar layer on a glass slide (in a microchamber according to Peshkov, 1955) showed that, after the addition of a norsulfazol solution (2 mg %), both spore germination and division of vegetative cells which had just grown out of them ceases completely. Meanwhile, this concentration of norsulfazol no longer has any inhibitory effect on microcolonies formed after several cell divisions (consisting of 15 or more cells).

In order to determine the degree of resistance to norsulfazol, the culture was plated on agar media covering a range of concentrations of this substance.

Table 1. Number of Cells (106 per ml) in Bacillus megatherium Cultures on Various Media

Media	Hours after inoculation							
	0	24	30	36	40	44	48	56
Synthetic	0.5	270	420	440	580	590	**600***	560
	0.6	310	480	**500***	**500**	490	380	260
10% meat water	0.5	320	350	380	390	**420***	400	370
	0.6	370	380	**390***	**390**	250	150	90
100% meat water	0.4	1350	1590	**1770***	1670	1580	1500	—
	0.5	1020	1520	1600	**1750***	1740	1680	—
	0.5	1260	1600	1640	**1820***	1810	1420	—
MPB	0.4	1260	**1670***	1280	1100	900	—	—
	0.5	1350	**1720***	1300	1120	530	—	—
	0.5	1300	**1600***	1220	1080	880	—	—

* Maximal number of cells

Table 2. Number of Spores (in % of Total Number of Cells) in Streaks on Agar Slants

Agar-containing media		Streak No.	Days				
			2	3	5	7	10
Synthetic	Sublayered	1—2	25	45	67	77	82
		3—4	58	83	87	88	91
	Control	1—2	0	13	15	36	63
		3—4	1	2	4	6	24
10% meat water	Sublayered	1—2	0	31	47	50	58
		3—4	35	82	82	87	87
	Control	1—2	0	16	25	55	78
		3—4	10	18	30	36	50
MPA	Sublayered	1—4	3	7	8	9	11
	Control	1—4	0	0	0.5	1.5	2

The degree of resistance to sulfamides depends on the composition of the media, since the presence of para-aminobenzoic acid, certain amino acids (methionine, serine), as well as purine and pyrimidine bases lowers the toxic effect of sulfamide preparations. Agar medium made with meat water was used in our experiments. Two to three parallel plates were set up for each concentration of norsulfazol. The number of colonies growing up was counted after three to four days.

Some experiments were set up in the following manner. Ten ml of nutrient agar was poured into each Petri dish measuring about 10 cm in diameter, and 5 ml of the same agar, but containing a spore suspension, was poured on top of this. When observed under the microscope at low magnifications, it could be established that mass germination of spores started after five hours following inoculation, while after 16–17 hours, microcolonies consisting of 20–30 vegetative cells formed.

At the proper moment, 5 ml more of agar, containing an amount of norsulfazol such that after its diffusion through the entire thickness of the agar the required concentrations of this substance would be produced, was poured on top of the plates. Some of the plates were poured at the fifth hour, while others—on the 16th hour following inoculation.

The results given in Table 3 show that not a single germinating spore tolerated more than 3 mg% norsulfazol, while a considerable part of the microcolonies consisting of vegetative cells developed into colonies visible with the naked eye at this concentration, and some of them grew even at 50 mg% norsulfazol.

These data, as well as direct microscopic observations, showed that young cells which have just grown out of spores are considerably more sensitive to the given sulfamide preparation than are mature vegetative cells.

When inoculations on agar were made according to the usual method, conflicting results were obtained: when inoculated with spores, the culture sometimes showed considerably greater sensitivity to norsulfazol (Table 4, Exp. I and II) than when inoculated with vegetative cells, while in other cases (Exp. III), its sensitivity remained the same.

We were able to note that the discrepancy in the results depended on the physical state of the agar: On freshly prepared agar, the spores swelled rapidly in the moisture covering it, and gave sprouts; on slightly dried agar, spore germination was delayed. Consequently, in the latter case, norsulfazol had started to act on the spores even before they began to germinate. It can be supposed that the cells have time to develop some protective mechanism against norsulfazol within themselves during this preparatory period.

In order to check the indicated hypothesis, a washed spore suspension was inoculated into flasks of meat water. Into part of the flask, a small, relatively harmless amount of norsulfazol — 2 mg% — was added. The state of the culture was controlled microscopically by means of periodically withdrawn samples. The spores began to give sprouts after two–three hours in experimental and control flasks. After 10–11 hours, the culture already consisted chiefly of young vegetative cells, frequently joined in pairs. After several hours more, the culture grew extensively, and chains and aggregates of 15–20 cells appeared in it. Inoculations of a range of agar media containing norsulfazol were made at the three intervals mentioned here.

As can be seen (Fig. 2), the spores which had germinated on medium without norsulfazol, as well as the young vegetative cells which had formed from them, were very sensitive to the given substance. Later, after several generations, the resistance of the vegetative cells increased considerably.

The preliminary treatment of spores with small doses of norsulfazol apparently causes corresponding adaptive-physiological reorganizations in them. These reorganizations are manifested by the fact that the young cells which grow out of such spores are no less resistant to norsulfazol than are mature vegetative cells in the control culture. We did not investigate what constitutes the defensive mechanism against norsulfazol. It possibly amounts to increased produc-

Table 3. Number of Colonies in Petri Dishes Which Had Been Layered with Agar Containing Various Amounts of Norsulfazol after 5 and 16 Hours Following Inoculation

Final concentration of norsulfazol, mg %	Poured at fifth hour			Poured at 16th hour		
	No. of colonies in individual expts.		% of control	No. of colonies in individual expts.		% of control
	experiments			experiments		
	I	II		I	II	
(Control)						
0	58	120	100	52	132	100
1	35	32	44	27	72	53
2	6	28	17	20	68	45
3	6	11	9	21	69	46
5	0	0	0	16	60	38
7	0	0	0	17	62	39
15	0	0	0	18	66	42
22	0	0	0	11	48	29
33	0	0	0	12	31	24
50	0	0	0	2	12	7

Table 4. Number of Colonies on Agar Containing Various Concentrations of Norsulfazol when Inoculated with Spores and Vegetative Cells

Concentration of norsulfazol, mg %	Inoculation with spores			Inoculation with a two-day vegetative culture		
	experiments			experiments		
	I	II	III	I	II	III
0	80	140	145	78	96	132
1	17	52	148	79	90	130
2	8	50	145	80	102	135
3	5	28	150	78	106	132
5	7	18	146	82	98	120
7	6	12	148	72	92	115
10	0	0	138	60	90	118
15	0	0	142	62	94	117
22	0	0	136	58	82	114
33	0	0	124	49	71	102
50	0	0	86	6	15	12

Note. Experiments I and II–inoculation on freshly-prepared agar; experiment III–inoculation on slightly dried agar.

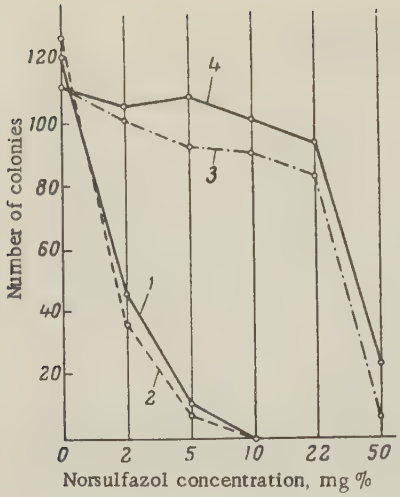

Fig. 2. The effect of the physiological age of a Bacillus megatherium culture on its resistance to norsulfazol. 1) Agar inoculated with germinating spores; 2) inoculation with young vegetative cells; 3) inoculation with mature vegetative cells; 4) inoculation with germinating spores placed into a 2 mg% norsulfazol solution three hours prior to germination.

tion of para-aminobenzoic acid, known as an antagonist of sulfamide preparations.

Resistance to norsulfazol, which arises in the process of vegetative reproduction in the absence of this substance, probably does not have such a specific character. As has already been stated at the beginning of the article, mature vegetative cells differ from young ones by their increased resistance to unfavorable effects of the most varied character—temperature, osmotic pressure, antiseptics, etc.

SUMMARY

The change in the relationship of Bacillus megatherium to conditions of the medium as it passes through the stages of its life cycle was studied. It was found that the transition from the vegetative state to sporulation is stimulated by the disappearance from the medium of nutrient substances necessary for the growth and reproduction of the cells. Using norsulfazol as an example, it was shown that germinating spores and the first cells which are formed from them have an increased sensitivity to toxic substances. During further vegetative reproduction, the resistance of the cells increases considerably. Preliminary treatment of spores with small doses of norsulfazol activates some defensive physiological mechanisms. Due to this, spores at the time of germination as well as the young cells which grow out of them exhibit as much resistance to the given substance as mature vegetative cells.

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PATHWAYS OF FORMATION AND TRANSFORMATION OF ORGANIC ACIDS BY ACTINOMYCES VIOLACEUS

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It is known that actinomycetes occur very widely in nature and are producers of antibiotic substances which vary greatly in structure; therefore, the study of the pathways of the transformation of carbon compounds by various species of these microorganisms is of great significance.

It seemed expedient to us to study the transformation of organic acids in one representative of the actinomycetes—Actinomyces violaceus—and to clarify the possible connection between these processes and antibiotic production, especially since this question has been studied little to date.

EXPERIMENTAL PART

The transformation of organic acids was studied on synthetic medium which contained the following mineral salts: 0.5 g/liter K_2HPO_4; 0.5 g/liter $MgSO_4$; 0.5 g/liter NaCl; 1 g/liter KNO_3; 2.5 g/liter $CaCO_3$; traces of iron. To this mineral medium we added pyruvic, malic, succinic, and citric acids in the form of their sodium salts in amounts of from 0.25 to 0.5%, each separately. The media were sterilized, and were then inoculated with A. violaceus spores. The inoculated media in flasks were placed on a shaker (120 rpm), and fermentation was carried out at 26–27°.

During the course of the process, 25-ml samples of culture fluid were collected, sulfuric acid was added to a pH of 2–3, and organic acids were extracted with diethyl ether in a Soxhlet apparatus using special flasks with funnels. The solutions of organic acids in diethyl ether obtained were concentrated by distilling off the ether. The organic acids were extracted from the residue in the flask with 2 ml of water five times. The aqueous solutions of organic acids were concentrated to 1 ml in a vacuum desiccator over sulfuric acid at room temperature.

In order to separate the organic acids in the aqueous solution which we obtained, we used the method of partition chromotography on paper. The test fluid was placed on No. 2 Leningrad mill paper in amounts of from 10 to 25 μ liters depending on the concentration of organic acids in the culture fluid being tested. We used water-saturated n-butanol with 5% formic acid as the solvent. The spots of the test acids were developed after spraying the paper with a 0.4% alcoholic solution of bromcresol green. Qualitative determinations of the acids were done by matching the R_f of the acids in the test fluids with the R_f of pure solutions of organic acids as is shown on the chromatograms.

THE TRANSFORMATION OF MALIC ACID

Fermentation by A. violaceus in the presence of sodium malate was carried on for three and six days. After this interval, the organic acids were extracted by the method given above and were chromatographed on paper (Fig. 1 and 2).

As seen from the chromatogram (Fig. 1), A. violaceus forms about seven acids from malic acid alone: glycolic, lactic, succinic, and fumaric. We have not yet been able to identify the other three acids. It is also seen from the chromatogram that A. violaceus does not decompose malic acid completely during three days of fermentation. Considerable amounts of it remained in the culture fluid. By the size of the spots, malic acid was prevalent over all the rest of the acids. At this stage of development, A. violaceus formed quite appreciable amounts of the above-mentioned acids from malic acid (Fig. 1).

During more prolonged A. violaceus fermentation (six days), malic acid was almost entirely assimilated. In this case, the composition of the newly formed acids also changed. Instead of the seven organic acids in the three-day culture, only four acids remained in the six-day culture: unknown, lactic, succinic, and fumaric. Moreover, lactic acid predominated over all the remaining acids in this period. This was clearly shown in the chromatogram (Fig. 2); the lactic acid spot was larger than the remaining acids in size.

In order to establish the fermentative conversions of malic and lactic acids, we studied the activity of the dehydrogenases of these acids.

Investigations showed that an acetone preparation of mycelium obtained from a three-day culture of A. violaceus in the presence of sodium malate and DPN^+ decolorized methylene blue in 65 minutes. No decolorization of methylene blue was observed in this interval of time in the absence of substrate. In the presence of sodium lactate and DPN^+, the acetone preparation also decolorized methylene blue in 85 minutes. These facts suggest that A. violaceus contains malic and lactic acid dehydrogenases.

THE TRANSFORMATION OF SUCCINIC ACID

Succinic acid was assimilated very vigorously by A. violaceus; its fermentation was carried out in three days. During this time, this microorganism produced only two acids from succinic: fumaric, and one acid which we have not yet been able to identify (Fig. 3).

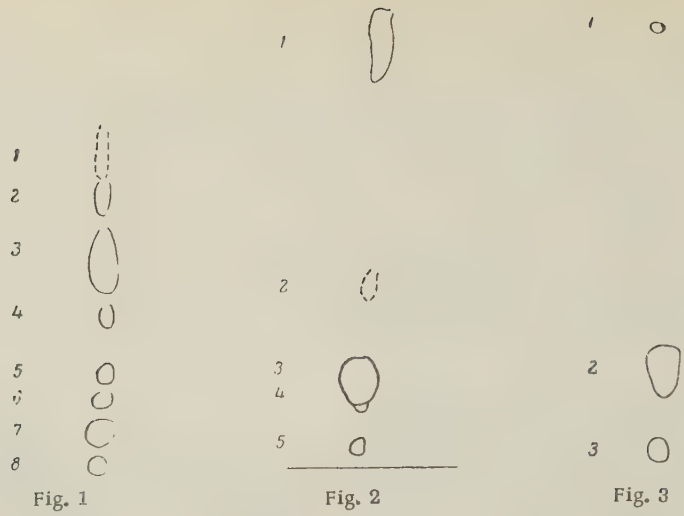

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Paper chromatogram of organic acids. Three-day culture (fermentation with sodium malate). 1-2) Unknown acids; 3) malic; 4) glycolic; 5) lactic; 6) succinic; 7) fumaric; 8) unknown.

Fig. 2. Paper chromatogram of organic acids. Six-day culture (fermentation with sodium malate). 1) Unknown; 2) malic; 3) lactic; 4) succinic; 5) fumaric.

Fig. 3. Chromatogram of organic acids. Three-day culture (fermentation with sodium succinate). 1) Unknown; 2) succinic; 3) fumaric.

A. violaceus apparently contains succinic dehydrogenase which dehydrogenates succinic acid to fumaric. This was confirmed during the study of the dehydrogenase activity of the acetone preparation of A. violaceus mycelium. The acetone preparation of mycelium decolorized methylene blue in 55 minutes in the presence of sodium succinate under anaerobic conditions. In one of the preceding works (Bekhtereva, 1960), we studied the aerobic oxidation of succinic acid. Washed A. violaceus mycelium oxidized succinic acid very vigorously. In the presence of sodium succinate, 100 mg of washed mycelium consumed 391 μ liters of oxygen in 90 minutes. When cytochrome was added to this system, the amount of oxygen consumed doubled.

Succinic acid was oxidized much more intensively during aerobic respiration than under anaerobic conditions. It is known that succinic dehydrogenase is part of the succinoxidase system, which also includes the cytochrome system.

Investigations which we conducted earlier on the anaerobic and aerobic respiration of A. violaceus showed that this microorganism contains the succinoxidase system, and that approximately 85% of its respiration is carried out by this system (Bekhtereva, 1960). A. violaceus gives excellent growth on Czapek's medium in the presence of succinic acid and produced antibiotic in amounts up to one million arbitrary units, while on the rest of the acids investigated, it produced smaller amounts of antibiotic under the same conditions.

THE TRANSFORMATION OF CITRIC ACID

The A. violaceus fermentation was carried out on medium with sodium citrate for three and six days. In the three-day culture, six acids were produced from citric acid alone: α-ketoglutaric, glycolic, lactic, succinic, fumaric, and one unknown (Fig. 4). During prolonged fermentation (six days), this microorganism assimilated nearly all of the citric acid added and all of the newly formed acids. On the sixth day of fermentation, three new acids were produced which were situated above citric acid on the chromatogram (Fig. 5). We have not yet identified these three acids. The organism which we investigated can not synthesize citric acid from pyruvic (Bekhtereva, 1960), but decomposes it intensively with the production of a number of organic acids with two or four carbon atoms.

As can be seen, A. violaceus contains enzymes which oxidize citric and isocitric acids. To confirm this assumption, we studied the activity of the dehydrogenases of these acids.

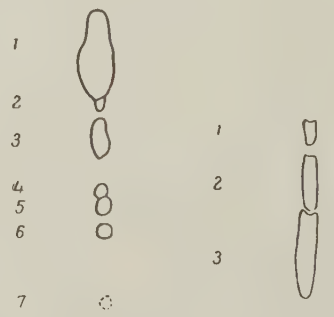

Fig. 4 Fig. 5

Fig. 4. Paper chromatogram of organic acids. Three-day culture (fermentation with citric acid). 1) Citric; 2) α-ketoglutaric; 3) glycolic; 4) unknown; 5) lactic; 6) succinic; 7) fumaric.

Fig. 5. Paper chromatogram of organic acids. Six-day culture (fermentation with citric acid). 1-3) Unknown acids. On the chromatogram, these acids are located above those found in the three-day culture and have a low R_f.

Experiments showed that the acetone preparation made from a three-day culture of A. violaceus decolorized methylene blue in the presence of isocitric acid and DPN$^+$ in 25 minutes, and in 45 minutes in the presence of citric acid. Methylene blue was not decolorized in this interval of time without the indicated substrates. Our test microorganism apparently contains an enzyme complex which oxidizes citric acid through cis-aconitic and isocitric to oxalosuccinic acid. The latter is rapidly decarboxylated to α-ketoglutaric acid which we always find during the chromatography of three-day culture fluid.

THE TRANSFORMATION OF PYRUVIC ACID

The A. violaceus fermentation on medium containing pyruvic acid was also carried out for three and six days. In the three-day culture, this organism formed seven acids in the presence of pyruvic acid: malic, glycolic, lactic, succinic, fumaric, and two unidentified acids (Fig. 6). In the six-day culture, the quantitative ratio of organic acids changed somewhat. A decrease in malic acid was observed with a simultaneous increase in glycolic (Fig. 7).

It is interesting to note that during the six-day fermentation (A. violaceus) in the presence of pyruvic acid, the composition of the newly formed acids changed little, while in the fermentation of medium containing sodium citrate, the complete assimilation of all organic acids formed in the early stage of growth of this microorganism was observed during this interval of time.

DISCUSSION OF RESULTS

The investigations carried out in order to study the transformations of organic acids by A. violaceus showed that this microorganism contains dehydrogenases and other enzymes which convert some acids to others by means of dehydrogenation, hydration, and decarboxylation. We showed by the paper chromatography method that A. violaceus growing on Czapek's medium without glucose can convert malic acid to succinic, fumaric, lactic, and glycolic. The oxidation of malic acid occurs by the following pathways. First, malic acid is dehydrated to fumaric, and the latter is reduced to succinic through the dicarboxylic acid cycle. On the other hand, in the presence of malic acid dehydrogenase and DPN$^+$, malic is dehydrogenated to oxalacetic. The latter is decarboxylated to pyruvic acid. In the presence of reduced DPN · H+H$^+$ and lactic dehydrogenase, the pyruvic acid formed is reduced to lactic acid.

In neutral medium, lactic dehydrogenase shifts the equilibrium in the direction of lactic acid production, which agrees well with our data. Actually, in a neutral medium at pH 7, relatively large amounts of lactic acid were produced from malic acid (Fig. 2).

As we have indicated above, A. violaceus did not synthesize citric acid from pyruvic and oxalacetic acids, but decomposed it vigorously with the production of α-ketoglutaric, succinic, and fumaric acids. It can be assumed that the microorganism contains an enzyme complex which oxidizes citric acid.

It is probable that at first, citric acid is converted to aconitic acid in the presence of aconitase. The aconitic acid is hydrated to isocitric. The isocitric acid formed is dehydrogenated to oxalosuccinic acid in the presence of dehydrogenase and DPN$^+$. Oxalosuccinic acid is rapidly decarboxylated to α-ketoglutaric. Oxalosuccinic acid is a very labile compound, and no one has yet been able to isolate it in biological media.

In a three-day culture of A. violaceus, traces of α-ketoglutaric acid were formed from citric acid (Fig. 4). The α-ketoglutaric acid can further be converted to succinic acid by means of oxidative decarboxylation.

A semialdehyde of succinic acid is probably formed as the intermediate product in this reaction.

The succinic acid formed is dehydrogenated to fumaric acid in the presence of succinic dehydrogenase. Thus, the formation of succinic and fumaric acids from citric can be explained from the above-mentioned reactions. As far as the formation of lactic acid is concerned, this is a very complex pathway. Citric acid is probably oxidized through the tricarboxylic acid cycle to oxalacetic, which is decarboxylated to pyruvic acid. The latter is reduced to lactic.

The production of lactic acid was well illustrated during the fermentation of pyruvic acid on Czapek's medium without glucose. In this case, as seen from the chromatograms (Fig. 6 and 7), from pyruvic acid alone this microorganism formed not only lactic, but malic, succinic, fumaric, and glycolic acids as well.

Pyruvic acid is the key intermediate product during fermentation and glycolysis in all microorganisms. At present, more than seven pathways of biochemical transformation of pyruvic acid are known. Of these, the most widely occurring is its decarboxylation by decarboxylase to acetaldehyde and CO_2 or carboxylation to oxalacetic acid by β-carboxylase. The oxalacetic acid formed is converted to malic, fumaric,

Fig. 6

Fig. 7

Fig. 6. Paper chromatogram of organic acids. Three-day culture (fermentation with sodium pyruvate). 1-2) Unknown; 3) malic; 4) glycolic; 5) lactic; 6) succinic; 7) fumaric.

Fig. 7. Paper chromatogram of organic acids. Six-day culture (fermentation with sodium pyruvate). 1-2) Unknown; 3) malic; 4) glycolic; 5) lactic; 6) succinic; 7) fumaric.

and succinic acids by means of hydrogenation and dehydrogenation in the dicarboxylic acid cycle. Another pathway is the production of acetyl-CoA from pyruvic acid by means of its decarboxylation to acetaldehyde and CO_2. In the presence of coenzyme and DPN^+, acetaldehyde is converted to acetyl-CoA. The condensation of two molecules of acetyl-CoA results in the formation of succinic acid. The latter, by way of the dicarboxylic acid cycle, is dehydrogenated by succinic dehydrogenase and is converted to fumaric acid. Fumaric acid is hydrated to form malic acid.

SUMMARY

1. Actinomyces violaceus grows on Czapek's medium in which glucose is replaced by organic acids (malic, succinic, citric, and pyruvic). However, the course of growth and antibiotic production is dissimilar. These processes go on most intensively on succinic acid. Antibiotic production is as high as one million arbitrary units during fermentation with succinic acid.

2. Under these conditions, A. violaceus oxidizes succinic acid more vigorously than the rest of the acids tested by us. The process of antibiotic production is probably associated with the intensity of oxidation–reduction reactions.

3. The transformation of organic acids during fermentation by A. violaceus in Czapek's medium was demonstrated by the paper chromatography method. This microorganism forms seven acids from malic acid: succinic, fumaric, lactic, glycolic, and three unidentified acids. Two acids–fumaric and an unidentified one–are formed from succinic acid.

4. A. violaceus does not synthesize citric acid from oxalacetic and pyruvic acids, but can oxidize it with the formation of seven acids: α-ketoglutaric, succinic, fumaric, lactic, glycolic, and two unidentified acids.

5. The conversion products of pyruvic acid were found to be: succinic, fumaric, malic, lactic, glycolic, and two unidentified acids.

6. The amount of acids formed changes during the course of fermentation. Thus, for example, seven acids were formed on the third day in medium containing citric acid, while on the sixth day, the composition of organic acids changed and three new acids were formed. Quantitative changes in the acids formed are also observed in medium with malic acid. The amount of newly formed acids decreased by the sixth day with the exception of lactic, the amount of which increased noticeably.

7. The transformation of the acids which we studied is evidence of the fact that A. violaceus contains enzyme systems which participate in oxidation–reduction processes and lead to the formation of new acids.

THE PHYLOGENETIC RELATIONSHIPS OF ACETIC ACID BACTERIA

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According to modern determinative keys of bacteria (Bergey, 1957; Krasil'nikov, 1950), acetic acid bacteria [genus Acetobacter (Beijerinck, 1898)] belong to the Pseudomonadaceae family.

Bacteria of the genera Acetobacter and Pseudomonas (Migula, 1900) were first united into a single family by Kluyver and van Niel (1936) on the basis of similar morphological characteristics, particularly polar flagellation and their physiological properties—heterotrophic nutrition and obligately oxidative energy metabolism.

Later studies of the physiology of the bacteria of the genera Acetobacter and Pseudomonas revealed many common properties in them. Thus, it was determined that most bacteria of the genus Pseudomonas, similarly to acetic acid bacteria, oxidize monosaccharides with the formation of hexonic and pentonic acids in large amounts (Pervozvanskii, 1939; Lockwood, Tabenkin, and Ward, 1941; Entner and Stanier, 1951). At the same time, it was found that some strains of Pseudomonas fluorescens oxidize gluconic acid to 2-ketogluconic and produce other keto acids, differing from acetic acid bacteria only in that the former act on the second carbon atom, while the latter act on the fifth, although it later became known that some acetic acid bacteria can also form 2-ketogluconic and even 2-5-ketogluconic acids (Katznelson, Tanenbaum, and Tatum, 1953).

In 1947, Stanier found that the oxidation of alcohol to acetic acid, which is so characteristic for bacteria of the genus acetobacter, is also characteristic for the genus Pseudomonas.

Recently, some investigators have disputed the unity of the genus Acetobacter and the phylogenetically close relationship of some of the bacteria in this genus to the bacteria of the Pseudomonadaceae family. In an article by Leifson (1954), the existence among acetic acid bacteria not only of mono- and lophotrichous organisms, but of peritrichous ones as well is reported. In the opinion of the author, different types of flagellation are correlatively associated with physiological types within the genus. On the basis of the fact that, according to Kluyver and van Niel, peritrichous bacteria and bacteria with polar flagella differ in origin, Leifson places lophotrichous bacteria which do not oxidize acetic acid in the genus Acetomonas in the Pseudomonadaceae family, and acetic acid bacteria which he regards as peritrichous or devoid of flagella —in the genus Acetobacter, which in his opinion belongs to a different family. Sharing this view, Shimwell (1957) considers it necessary to recognize the existence of the family Acetobacteriaceae with one genus—Acetobacter.

Leifson's data on different types of flagellation among acetic acid bacteria require serious examination, especially since the photographs which he gives in his article are not very convincing.

In connection with the above-stated, the necessity arises for a comparative study of the morphology and physiology of the bacteria in the genus Acetobacter and of the most typical representatives of the Pseudomonadaceae family—bacteria of the genus Pseudomonas. Further investigations will show whether there is any basis for relating acetic acid bacteria not only to two different genera, but even to two different families as proposed by Leifson and Shimwell, and whether, as proposed by Frateur (1950) and by Janke (1957), the "suboxydans" group of lophotrichous bacteria which does not oxidize acetic acid is actually a more primitive form and is directly related to the genus Pseudomonas.

In the present communication, we present some results of a comparative study of the morphological and physiological properties of bacteria of the genera Acetobacter and Pseudomonas.

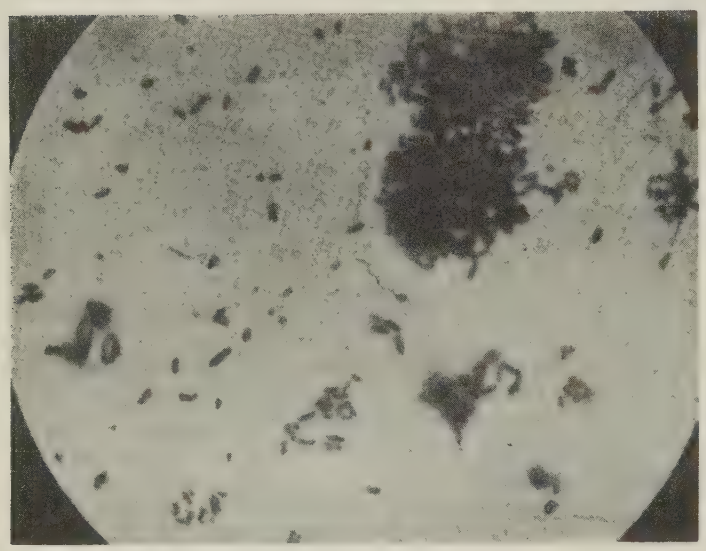
We had the following four cultures of bacteria of the genus Acetobacter at our disposal: A. aceti (three strains of bacteria used in the rapid method of vinegar production), A. xylinum, A. suboxydans (two strains) and A. melanogenum, and 24 cultures of bacteria of the genus Pseudomonas. We selected the latter from 83 cultures of this genus on the basis of their ability to oxidize ethyl alcohol; these cultures were kindly made available to us by colleagues at the Institute of Microbiology of the Academy of Sciences, USSR, the Institute of Agricultural Microbiology VASKhNIL and its Moscow branch, the Soils Museum of the Academy of Sciences, USSR, and the departments of microbiology of Moscow State University and Leningrad State University. We should like to take this opportunity to express our gratitude to the above institutions.

Among these cultures, strains of the following species of Pseudomonas were found: P. fluorescens, P. liquefaciens, P. nonliquefaciens, P. denitrificans, P. mycophage, P. radiobacter, P. pyocyaneum, P. gracilis, and several cultures for which the species relationship was not determined.

While investigating the morphology of the bacteria, we devoted particular attention to the determination of cell size and the conditions optimal for their reproduction, staining of the flagella, the determination of their length, and the Gram stain.

Table 1. The Morphological Characteristics of Bacteria of the Genera Acetobacter and Pseudomonas

Species of bacteria	Dimensions of cells in μ	Type of flagellation	Length of flagella in μ
Acet. suboxydans	1.5—1.8×0.5	Lophotrichous	4.5
" melanogenum	1.8—2.0×0.6	Lophotrichous	—
" aceti	1.2—1.5×0.4	Monotrichous	11.7
" xylinum	2.0—2.4×0.5	Nonmotile	—
Ps. fluorescens	2.7—3.3×0.6	Monotrichous	6.8
" liquefaciens	3.9—4.0×0.5	Monotrichous	—
" nonliquefaciens	3.8 ×0.5	Mono- and lophotrichous	4.5
" denitrificans	1.8—2.2×0.5	Monotrichous	—
" gracilis	2.0—2.4×0.5	Lophotrichous	4.6



Flagella of Acetobacter aceti.

Table 2. Some Cultural Characteristics of Bacteria of the Genera Acetobacter and Pseudomonas

Species of bacteria	Growth on synthetic media	Milk		Wort-gelatin with alcohol		Peptone agar		Yeast agar with glucose		Medium with $NaNO_3$, NO_2 production
		growth	peptonization	growth	liquefaction	growth	pigment	growth	pigment	
Acet. suboxydans	—	—	—	—	—	—	—	+	—	—
" melanogenum	—	—	—	—	—	—	—	+	+	—
" xylinum	—	—	—	+	—	—	—	+	—	—
" aceti	+	—	—	+	—	—	—	+	—	—
Ps. fluorescens	+	+	+	+	+	+	+	+	+	+
" liquefaciens	+	+	+	+	+	+	+	+	—	—
" nonliquefaciens	+	+	+	+	—	+	—	—	—	—
" pyocyaneum	+	+	+	+	+	+	+	+	+	+
" radiobacter	+	+	+	+	—	+	—	+	—	+
" denitrificans	+	+	—	+	—	+	—	+	—	+
" gracilis	+	+	—	+	—	+	—	+	—	—
" mycophaga	+	+	—	+	—	+	—	+	—	+

Some of the data of the morphological observations are given in Table 1.

All of the bacteria studied were relatively small Gram-negative nonsporogenous rods. The ratio of length to width was somewhat higher in Pseudomonas species than among acetic acid bacteria. We should like to draw attention to the fact that all of the bacteria, both those belonging to the genus Pseudomonas and those of the genus Acetobacter (except for the nonmotile A. xylinum) proved to be mono- or lophotrichous.

According to Leifson's data, all motile acetic acid bacteria which oxidize acetic acid to carbon dioxide and water are peritrichous including A. orleanense, A. rancens, and A. aceti.

Our investigations did not confirm this. All strains of A. aceti which oxidize acetic acid to CO_2 and H_2O were found to be monotrichous (see figure). For flagellar strains, the bacteria were cultured in alcohol-containing synthetic medium from which they were transferred directly to a glass slide. Staining was carried out according to the Loeffler method as modified by Culp. In his work, Leifson mentions that when staining flagella according to his method, centrifugation and washing of bacteria grown in glucose-containing nutrient medium was carried out. As a consequence of this treatment, a phenomenon similar to agglutination (formation of clumps) was observed. It is especially difficult to obtain a good preparation by this method, which would demonstrate the true arrangement of bacterial flagella. The peritrichous flagellation of A. aceti on Leifson's microphotograph is so indistinct that it can be regarded as an artifact.

Data on the cultural characteristics of some of the cultures which we studied are given in Table 2. Nitrate reduction, growth in milk, growth and pigment production on peptone agar, growth and gelatin liquefaction (in wort), pigment production on yeast agar with chalk, and growth in alcohol-containing synthetic medium were studied. Synthetic medium of the following composition was used: $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4$ in 0.1% amounts in tap water. The bacteria were grown under conditions of "aerial" nutrition—the ethyl alcohol was added to the dessicator where the inoculated flasks or test tubes were placed. This method has been described more thoroughly in an article by Loitsyanskaya (1959).

The principal characteristics recommended by Frateur for studying the properties of acetic acid bacteria were selected for studying the physiology of the bacteria of the genera Acetobacter and Pseudomonas.

1. Utilization of ethyl alcohol in synthetic and energy metabolism and acetic acid accumulation in Hoyer's and Frateur's media and in yeast water, as well as on yeast agar with chalk and on Frateur's medium with agar and chalk.
2. Utilization of acetic acid and its salts in synthetic and energy metabolism.
3. Oxidation of calcium lactate (by Frateur's method).
4. The production of acids during the utilization of the following compounds: arabinose, glucose, fructose, galactose, sucrose, maltose, lactose, and raffinose.
5. The formation of keto compounds—dioxyacetone from glycerol, calcium ketogluconate from calcium gluconate, sorbose from sorbitol, fructose from mannitol (by the oxidogram method according to Frateur and by determination of the amount of reduced copper).
6. The presence of catalase.

In view of the limited length of this article, we can not present the numerical materials which we obtained here. We shall therefore limit ourselves to only two summary tables in which data are given which reflect the similarity and uniqueness of physiology of acetic acid bacteria and bacteria of the genus Pseudomonas. In these tables (for the sake of brevity), we do not present the characteristics of all of the bacterial cultures of the genus Pseudomonas studied (there were 24 of them), but only of the representatives of five species of this genus.

As is well known, the oxidation of alcohol to acetic acid is a characteristic property of the genus Acetobacter. However, different species of this genus carry out this process at different rates. The bacteria of the rapid method for vinegar manufacture, A. aceti, oxidize alcohol to acetic acid the most actively (Loitsyanskaya, 1959a).

Of the 83 bacterial cultures of the genus Pseudomonas tested by us, 24 possessed the ability to oxidize alcohol to acetic acid. On solid nutrient medium containing chalk, zones where chalk was dissolved were observed around colonies of these bacteria, which indicates acid production. However, when the bacteria grew in liquid nutrient medium with alcohol, acetic acid did not accumulate, since all strains of Pseudomonas at our disposal actively oxidized alcohol and acetic acid to CO_2 and water.

A. aceti, A. xylinum, and all species of Pseudomonas used in the work grew in synthetic medium with alcohol as the carbon source both under conditions of "aerial" nutrition and when alcohol was added directly to the nutrient medium. The bacteria of the "suboxydans" group which we studied did not grow under conditions of "aerial" nutrition with ethyl alcohol. They cannot utilize ethyl alcohol in synthetic metabolism. We regard this as a substantial distinction in the physiology of the "suboxydans" group of bacteria from Pseudomonas and the "mesoxydans" group of acetic acid bacteria.

Not all acetic acid bacteria can utilize acetic acid and its salt, which is of diagnostic significance. The suboxydans group of bacteria does not utilize acetic acid and its salts. They do not "overoxidize" alcohol, i.e., they do not oxidize the acetic acid which they produce to carbon dioxide and water, which is actively carried out by the bacteria of the rapid method of vinegar manufacture, especially under conditions which are optimal for reproduction. Acetic acid salts are also utilized by the bacteria of the rapid method of vinegar manufacture; however, this utilization is much poorer than that of alcohol or of acetic acid.

Bacteria of the genus Pseudomonas can also utilize acetic acids salts as a carbon source, and oxidize them to carbon dioxide and water.

For the bacteria of the rapid method of vinegar manufacture, lactic acid, and especially its salts, are the best source of carbon. These bacteria, as well as some

Table 3. Utilization of Ethyl Alcohol, Glucose, and Acetic and Lactic Acid Salts by Bacteria of the Genera Acetobacter and Pseudomonas

Species of bacteria	Utilization in synthetic metabolism			Oxidation			
	ethyl alcohol	Ca acetate	glucose	ethyl alcohol	Ca acetate	Ca lactate	glucose
Acet. suboxydans	—	—	+	+	—	—	+
" melanogenum	—	—	+	+	—	—	+
" xylinum	+	+	+	+	+	+	+
" aceti	+	+	—	+	+	+	+
Ps. fluorescens	+	+	+	+	+	+	+
" liquefaciens	+	+	+	+	+	+	+
" nonliquefaciens	+	+	+	+	+	+	+
" pyocyaneum	+	+	+	+	+	+	+
" radiobacter	+	+	—	+	—	+	—
" denitrificans	+	+	+	+	—	+	+
" gracilis	+	+	+	+	—	+	+
" mycophaga	+	+	+	+	—	+	+

Table 4. Production of Acids and Keto Compounds During the Oxidation of Carbohydrates and Polyatomic Alcohols by Some Species of the Genera Acetobacter and Pseudomonas

Species of bacteria	Production of acids from								Prod. of keto compounds from			
	arabinose	glucose	galactose	fructose	sucrose	lactose	maltose	raffinose	glycerol	sorbitol	mannitol	Ca-gluconate
Acet. suboxydans	+	+	+	+	+	—	—	—	+	+	+	+
" melanogenum	+	+	+	?	+	—	—	—	+	+	+	+
" xylinum	+	+	+	+	+	—	—	—	+	+	+	+
" aceti	+	+	+	?	+	—	—	—	+	+	+	+
Ps. fluorescens	+	+	+	—	—	—	—	—	+	+	+	+
" liquefaciens	+	+	+	—	—	—	—	—	+	—	+	+
" nonliquefaciens	+	+	+	—	—	—	—	—	+	—	+	+
" pyocyaneum	+	+	+	—	—	—	—	—	+	—	+	+
" radiobacter	+	+	—	—	—	?	—	—	—	—	—	+
" denitrificans	+	+	+	—	—	—	—	—	—	+	+	+
" gracilis	+	+	+	—	—	—	—	+	—	+	+	+
" mycophaga	+	+	+	—	—	—	—	—	—	?	+	+

of the bacterial cultures of the genus Pseudomonas which we studied, actively oxidize Ca lactate. In distinction from these, the bacteria of the suboxydans group do not oxidize lactic acid salts to carbon dioxide and water (Table 3).

On the basis of the data given in Table 3, it can not be concluded that the bacteria of the suboxydans group have more physiological properties in common with bacteria of the genus Pseudomonas than do bacteria of the mesoxydans group. On the contrary, the great similarity of the mesoxydans group of bacteria with those of the genus Pseudomonas should be noted.

All of the acetic acid bacteria studied in the given work can oxidize aldoses to the corresponding acids. This is also characteristic for bacteria of the genus Pseudomonas. Of the disaccharides, acetic acid bacteria oxidize only sucrose, forming an insignificant amount of acid. Bacteria of the genus Pseudomonas do not form acids from disaccharides (Table 4).

The production of keto compounds is a characteristic property of some species of acetic acid bacteria. We detected the ketogenic ability of the test cultures by the oxidogram method proposed by Frateur. Acetic acid bacteria were grown on yeast agar with glycerol, while bacteria of the genus Pseudomonas were grown on meat-peptone agar with glycerol. Aside from this, all bacteria were grown in yeast water with glycerol and with sorbitol. In the latter case, we obtained comparative data on the formation of dioxyacetone and sorbose by different bacterial cultures by means of determining the amount of reduced copper in Fehling's solution. These were long-lasting experiments – 1½ months.

We established that many bacteria of the genus Pseudomonas produce keto compounds; however, all of the acetic acid bacteria which we studied carried this out considerably more actively.

All of the cultures of bacteria of the genera Acetobacter and Pseudomonas used in the work had active catalase.

The systematics of acetic acid bacteria is still very incomplete. To date, various authors have described more than 35 species of acetic acid bacteria. It can be said with certainty that many of them are not real species, but are only varieties or variants of several distinct species. The extreme view on this question was expressed by Shimwell, who feels that only two species exist within the genus Acetobacter: A. aceti and A. xylinum. In Shimwell's opinion, all the rest of the acetic acid bacteria are mutants of these species; in this way, the extremely wide variability of the morphological and physiological properties of the bacteria in this genus is recognized.

In the discussion of the origin of acetic acid bacteria by a number of investigators, the opinion is expressed that bacteria of the peroxydans, oxydans, and mesoxydans groups are of different origin than the suboxydans group, the representatives of which are directly related to the genus Pseudomonas. It is precisely on this basis that it has been proposed that bacteria belonging to suboxydans be separated into an independent genus, Acetomonas, in the Pseudomonadaceae family, while bacteria of the genus Acetobacter be excluded from this family (Leifson, Shimwell, Janke).

The data which we obtained on the comparative study of the morphology and physiology of certain representatives of the genus Acetobacter and of bacteria of the genus Pseudomonas provide a basis for assuming that the bacteria of the rapid method of vinegar manufacture—A. aceti (Beijerinck) and A. xylinum (Brown), which are the mesoxydans group according to Frateur—are phylogenetically closer to bacteria of the genus Pseudomonas than is the suboxydans group.

The materials on the evolution of acetic acid bacteria now available are obviously inadequate for responsible generalizations. However, there are reasons to suppose, as has already been stated by Kluyver and van Niel, that bacteria of the genus Acetobacter and bacteria of the genus Pseudomonas had a common ancestor. Common traits of cell morphology of the bacteria in these two genera, particularly polar flagellation (the majority of species of acetic acid bacteria are mono- and lophotrichous) and the great similarity of their physiology—obligate aerobiosis and the capacity for oxidative transformations—are evidence of this.

The evolutionary pathways of the bacteria which were ancestors of the Pseudomonadaceae family were naturally diverse, which led to the rise of many genera in this large family and, specifically, of Acetobacter and Pseudomonas. The separation of the genera Acetobacter and Pseudomonas was accompanied in the first of these by increased oxidative ability and, in connection with this, by adaptation to growth in acid medium, while in the second—by the intensification of the ammonifying function, development of proteolytic enzyme systems, and adaptation to existence in alkaline medium.

Present-day acetic acid bacteria differ from bacteria of other physiological groups by a higher activity in the oxidation of various alcohols, carbohydrates, saccharocarbonic acids, cyclic polyatomic alcohols, and many other nonnitrogenous compounds. Acids are frequently encountered among the products of the vital activity of acetic acid bacteria, which explains the fact that many of them have adapted to growth in acid medium.

We see the genus Acetobacter as a single one. Naturally, different species of this genus differ substantially one from another in their physiological and ecological properties. The materials available on the phylogeny of acetic acid bacteria confirm that they belong to the Pseudomonadaceae family.

SUMMARY

1. The polar attachment of flagella in motile acetic acid bacteria, as well as the ability of active oxidative transformations and of growth in acid media, indicate that all acetic acid bacteria belong to one genus, viz., Acetobacter. Hence, the division of this genus into two genera, Acetomonas and Acetobacter, as suggested by Leifson, is unfounded.

2. Active consumption by the cultures of the Pseudomonas genus of ethanol and of acetates as a carbon source in a synthetic nutrient medium with a mineral source of nitrogen, oxidation of Ca lactate, and in some cases of acetic acid, point to a great similarity in the physiology of bacteria of the Pseudomonas genus and of acetic acid bacteria of the "mesoxydans" group (Frateur) and hence suggest a phylogenetic relationship between these bacterial forms.

3. The uniformity in cell shape and size of bacteria of the Acetobacter and Pseudomonas genera, their similarity in Gram-negative staining, similar flagellar pattern, the ability to oxidize ethanol into acetic acid and the ability to oxidize carbohydrates and polyatomic alcohols—all these facts are indicative of a close phylogenetic relationship between the Acetobacter and Pseudomonas genera and warrant the conclusion that they belong to the same Pseudomonadaceae family.

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THE EFFECT OF THE CALCIUM ION ON THE NITROGEN AND PHOSPHORUS METABOLISM OF AZOTOBACTER VINELANDII

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Some investigators maintain that calcium is necessary only for nitrogen fixation, and that its presence in the medium is not required when growing azotobacter on bound nitrogen (Burk and Lineweaver, 1931; Schroder, 1932, Horner and Burk, 1934; Bortels, 1936; Radler, 1955; Esposito and Wilson, 1956a, 1958).

However, other authors adhere to the opposite view, believing that calcium is equally necessary for the growth of azotobacter both on free and on bound nitrogen (Birch-Hirschfeld, 1932; Burk and Horner, 1940; Burk and Burris, 1941; Jensen, 1954; Norris and Jensen, 1957, 1958).

Norris and Jensen (1958), who studied the effect of calcium on the growth of a large number of strains of different azotobacter species on molecular and ammonium nitrogen, came to the conclusion that the divergence in the conclusions of these two groups of authors can apparently be explained by different amounts of inoculum as well as strain differences in azotobacter. The ability of different species of azotobacter to adapt to the absence of calcium in the medium at unequal rates can possibly also play an important role in the given phenomenon.

Esposito and Wilson (1956a), who studied the effect of calcium and phosphorus concentration in the medium on the growth of Azotobacter vinelandii, found that calcium is in some way associated with phosphorus metabolism. Somewhat later, these same authors established a relationship between the quantitative content of calcium in the medium and the extent of polyphosphate synthesis in A. vinelandii (Esposito and Wilson, 1956b). The fact observed by Esposito and Wilson that nitrogen fixation is inhibited in the absence of calcium along with the cessation of polyphosphate synthesis made it possible for them to assume that polyphosphates are necessary as an energy source for nitrogen fixation (Esposito and Wilson, 1956b). There are indications in the literature that calcium uncouples oxidation and phosphorylation in plant (Jagendorf and Arnon, 1959) and animal tissues (Slater and Cleland, 1952, 1953). Azotobacter requires calcium during oxidative phosphorylation (Hovenkamp, 1958). In the given microorganism, calcium can evidently replace magnesium in this process.

In the present work, we attempted to clarify the effect of the calcium ion in the medium on phosphorus and nitrogen metabolism of A. vinelandii grown on molecular and ammonium nitrogen.

MATERIALS AND EXPERIMENTAL METHODS

It seemed expedient to us to follow the course of changes in certain nitrogenous and phosphorus compounds in A. vinelandii under normal conditions and in the absence of calcium from the medium at a time when the cells, after prolonged phosphorus starvation, had the capacity to accumulate relatively large amounts of this element.

For this purpose, a bacterial mass grown on Burk's medium was centrifuged and transferred to phosphorus-free medium. As shown by preliminary experiments, a considerable phosphorus-impoverishment of the culture could already be observed after 24 hours. The phosphorus-deficient azotobacter cells were then placed in a medium with an increased phosphorus content (by 25%) with (normal) and without calcium, respectively, where they were enriched with phosphorus during 5–6 hours.

Similar experiments were carried out with an A. vinelandii culture grown on molecular and on ammonium nitrogen in order to compare the effect of the calcium ion on phosphorus and nitrogen metabolism and, particularly, on nitrogen fixation.

On the basis of the data of the experiments described above, we noticed that when azotobacter was placed in phosphorus-containing medium, the phosphorus-deficient culture accumulated basic phosphorus compounds at the greatest rate in the first two hours, after which their content in the cells changed very little. In view of this, we carried out the enrichment of phosphorus-deficient cells for 2.5 hours without calcium and then transferred them to a medium containing calcium and phosphorus. In order to follow the course of enrichment of the cells with phosphorus, samples were taken after 20, 40, and 60 minutes. In order to determine the points at which the absence of the calcium ion affects phosphorus metabolism, the phosphorus–enrichment of the cells was carried out in medium without calcium and with P^{32}, after which the cells were transferred to complete medium without radiophosphorus.

Analyses of the different forms of nitrogen and phosphorus, as well as radioactivity measurements of the latter were carried out by methodological procedures which we described earlier (Belozerskii, Zaitseva, Gavrilova, and Mineeva, 1957; Zaitseva and Belozerskii, 1958; Zaitseva, Belozerskii, and Novozhilova, 1959).

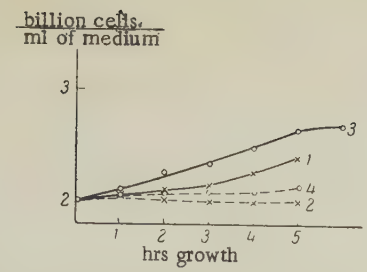

Fig. 1. Effect of the calcium ion on the growth of Azotobacter vinelandii during the enrichment of cells with phosphorus. Designation of curves in Fig. 1: 1) Cells grown on molecular nitrogen in the presence of calcium (on N_2 with Ca^{++}); 2) cells grown on molecular nitrogen in the absence of calcium (on N_2 without Ca^{++}); 3) cells grown on ammonium nitrogen in the presence of calcium (on NH_4^+ with Ca^{++}); 4) cells grown on ammonium nitrogen in the absence of calcium (on NH_4^+ without Ca^{++}).

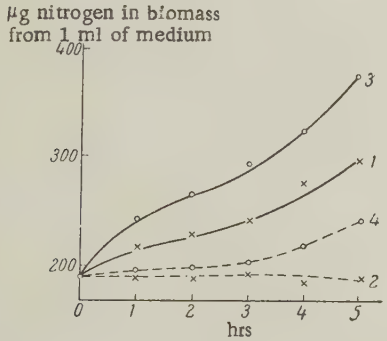

Fig. 2. Effect of the calcium ion on nitrogen fixation and assimilation of ammonium nitrogen from the medium by azotobacter. Curve designations same as in Fig. 1.

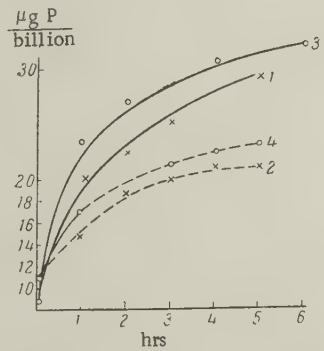

Fig. 3. Effect of calcium ion on the total phosphorus content of azotobacter cells. Designations of curves same as in Fig. 1.

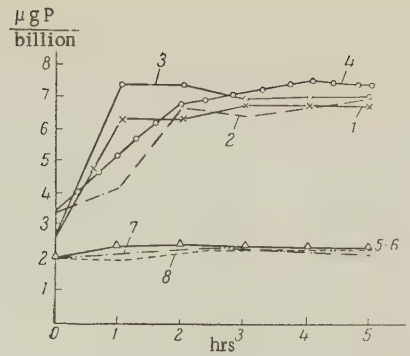

Fig. 4. Accumulation of ribonucleic (RNA) and desoxyribonucleic (DNA) acids by azotobacter as a function of the calcium concentration of the medium. 1-4) RNA nitrogen (designations same as in Fig. 1); 5-8) DNA nitrogen; 5) on N_2 with Ca^{++}; 6) on N_2 without Ca^{++}; 7) on NH_4^+ with Ca^{++}; 8) on NH_4^+ without Ca^{++}.

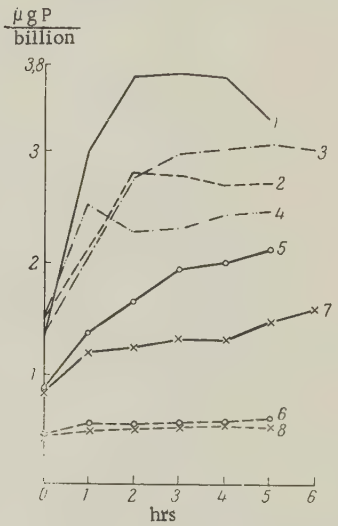

Fig. 5. Effect of calcium ion on the content of intracellular phosphorylated polysaccharides and phospholipids in azotobacter. 1-4) Polysaccharide phosphorus; 5-8) phospholipid phosphorus (designations same as in Fig. 1).

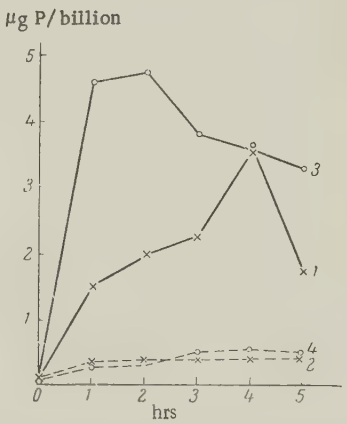

Fig. 6. The accumulation of polyphosphates in azotobacter as a function of the calcium content of the medium. Designations of curves same as in Fig. 1.

EXPERIMENTAL RESULTS

In studying the effects of calcium on the growth of azotobacter in the presence of molecular and of ammonium nitrogen, we came to the conclusion that the effect of calcium on nitrogen fixation is not specific.

This follows from the fact that the absence of calcium depressed the growth of both the nitrogen-fixing culture and the culture with inhibited nitrogen fixation equally (Fig. 1). If these data are compared with the content of total nitrogen, calculated on the basis of biomass in these two cultures, the conclusion which we arrived at becomes even more certain (Fig. 2).

Esposito and Wilson (1958) discovered that acetate can relieve the calcium requirement of A. vinelandii to some extent when it is grown on molecular nitrogen. A special experiment which we did to determine the role of citrate in the effect of calcium on growth and nitrogen fixation by A. vinelandii showed that the citrate ion does not replace the calcium ion which is essential for the normal growth of the nitrogen-fixing culture*.

It is seen from Fig. 3 that the absence of calcium ion had a very substantial effect on the quantitative content of total phosphorus in the azotobacter cell.

On the basis of the results obtained, it is natural to suppose that the primary effect of calcium is on some specific steps of phosphorus metabolism, and then, through it, on nitrogen metabolism as well.

When the data in Table 1 are examined, it is seen that the absence of calcium in the nitrogen-fixing culture caused decreased assimilation of orthophosphate from the medium and a sharp reduction in the accumulation of acid-soluble polyphosphates, as well as of labile forms of phosphorus. The amount of total stable phosphorus in the acid-soluble fraction (Table 1) and of nucleic acid phosphorus (Fig. 4) in this culture was essentially independent of the presence of calcium in the medium.

A deficiency of calcium ion in the medium also had a substantial effect on the process of phosphorylation of cellular polysaccharide and caused some reduction in phospholipid phosphorus (Fig. 5); this effect was most acutely reflected in the synthesis of polyphosphates (Fig. 6).

Similar results on the quantitative dynamics of different forms of phosphorus were also obtained for the culture grown on ammonium nitrogen with various contents of calcium in the medium (Table 1, Fig. 1–6).

Upon examination of the experimental data, it can be concluded that:

1) When there is a deficiency of calcium ion, the assimilation of orthophosphate from the medium decreases sharply. This leads to the fact that, under the given conditions, no storage of phosphorus in the form of polyphosphates occurs in the cell.

2) Calcium is essential for the synthesis of labile acid-soluble phosphorus compounds which, in turn, participate in the production of polyphosphates.

3) The synthesis of stable acid-soluble phosphorus compounds, consisting predominantly of phosphorylated carbohydrates and nucleoside monophosphates, is essentially independent of the presence of calcium ion in the medium.

4) During the phosphorus-enrichment of azotobacter cells, the rate of increase and the amount of RNA changes very little in relation to the calcium content of the medium (Fig. 4). The sharp reduction in polyphosphate synthesis during the relatively high rate of de novo formation of RNA in azotobacter cells in the absence of calcium is evidence of the fact that, during the enrichment of cells with phosphorus, the pathways of synthesis of polyphosphates and RNA may not be interrelated.

5) The quantitative content of DNA in the azotobacter cell does not depend on the calcium ion (Fig. 4).

In order to make these conclusions more precise, special experiments were set up with a more detailed interpretation of the quantitative changes in the labile and stable acid-soluble forms of phosphorus in azotobacter in relation to the calcium content of the medium (Tables 2 and 3). It is pertinent to note that detailed investigations (Zaitseva, Belozerskii, and Novozhilova, 1960) of the labile phosphorus of acid-soluble nucleotides in A. vinelandii during the stationary growth phase showed that more than 60% of the phosphorus of these compounds belongs to the adenylic system. This enabled us to conditionally name the labile phosphorus components of the mononucleotide fraction the "ADP – ATP" system.

We see from the data in Table 2 that the absence of calcium from the medium had a much greater effect on the content of "ATP – ADP" and polyphosphates in the cell ("ADP – ATP" decreased to 1/4, while polyphosphates decreased to 1/10) than on other phosphorus compounds (particularly on RNA and DNA). This is evidence of the fact that the formation of ATP is impaired in the absence of calcium, as the result of which the accumulation of reserve amounts of phosphorus in the form of polyphosphates does not occur in the azotobacter cell. At the same time, phosphorus metabolism proceeds along the basic energy pathway with the formation primarily of nucleic acids.

It has been shown in a number of works that the calcium ion is essential for respiration (Roberts and Rakestrow, 1956; Bullen and Frear, 1957). Since nitrogen fixation also does not occur in the absence of calcium (Fig. 2), it is natural to conclude that respiratory energy is necessary for nitrogen fixation. As we have already pointed out, the calcium concentration of the medium also affects the total phosphorus content in the azotobacter cell (Fig. 3).

From this it can be assumed that, in this microorganism, calcium is required to bring about certain oxidation – reduction reactions connected with respiration, as the result of which ATP is regenerated. This is confirmed by Hovenkamp's data (1958) on the requirement of calcium for oxidative phosphorylation in azotobacter.

* Due to the fact that the results obtained for the determination of the effect of the Ca^{++} ion on nitrogen and phosphorus metabolism in A. vinelandii were identical in the absence and in the presence of citrate, the data of the latter experiment are not presented in the work.

Table 1. Phosphorus-Enrichment of Azotobacter vinelandii Cells on Molecular and Ammonium Nitrogen in the Presence and in the Absence of Calcium in the Medium $\left(\text{in } \frac{\mu g\ P}{\text{billion cells}}\right)$

Time in hours	With Ca^{++}											Without Ca^{++}				
	Acid-soluble fraction															
	ortho-phosphate	soluble polyphosphate P (Ba precipitate)	labile P of the fraction	stable P of the fraction	total P of the fraction	Mononucleotide P			P of organic phosphoric esters of sugars			ortho-phosphate	soluble polyphosphate P (Ba precipitate)	labile P of the fraction	stable P of the fraction	total P of the fraction
						total	labile	stable	total	labile	stable					
On molecular nitrogen																
Initial phosphorus-deficient cells	0.60	0.58	0.71	1.02	2.33	0.34	0.03	0.31	0.81	0.10	0.71	0.62	0.44	0.63	1.29	3.38
Enriched																
1	1.82	0.41	0.77	2.11	4.70	0.42	0.11	0.31	2.05	1.80	1.27	1.27	—	0.47	2.80	4.54
2	2.25	0.54	1.03	3.53	6.27	1.11	0.19	0.92	2.91	0.30	2.61	1.75	0	0.4	3.18	5.47
3	2.20	0.78	1.35	3.98	7.53	1.37	0.23	1.14	3.18	0.34	2.84	1.92	0	0.57	3.73	6.22
4	2.31	1.30	2.10	4.22	8.63	1.40	0.30	1.10	3.62	0.50	3.12	2.12	0	0.82	3.94	6.86
5	2.64	2.22	2.90	3.80	9.34	1.48	0.41	1.07	3.00	0.27	2.73	2.15	0	0.88	3.97	7.00
On ammonium nitrogen																
Initial phosphorus-deficient cells	0.60	0.58	0.71	1.02	2.33	0.34	0.03	0.31	0.81	0.10	0.71	0.62	0.44	0.63	1.29	3.38
Enriched																
1	2.18	0.41	0.77	2.07	5.02	0.51	0.18	0.33	1.92	0.18	1.74	1.37	0.20	0.63	2.74	4.74
2	2.44	0.38	0.82	2.49	5.75	0.53	0.17	0.36	2.40	0.27	2.13	1.92	0.12	0.57	3.42	5.90
3	2.64	1.64	2.21	3.22	8.07	1.05	0.25	0.80	2.74	0.32	2.42	2.27	traces	0.60	3.79	6.66
4	2.80	1.89	2.61	4.03	9.44	1.60	0.35	1.25	3.15	0.37	2.78	2.34	0	0.42	4.38	7.17
5	2.73	2.26	3.10	4.21	10.04	1.74	0.38	1.36	3.31	0.46	2.85	2.51	0	0.33	4.55	7.39
6	2.56	3.60	4.40	4.21	11.17	1.83	0.46	1.37	3.18	0.34	2.84	—	—	—	—	—

Table 2. Quantitative Content of Some Phosphorus Compounds During the Phosphorus Enrichment of Azotobacter in the Presence and in the Absence of Calcium in the Medium $\left(\text{in } \frac{\mu gP}{\text{billion cells}}\right)$

Conditions and time of enrichment	Acid-soluble fraction		Conditions and time of enrichment		Acid-insoluble fraction		
	"ATP-ADP" ~P, mononucleotides	total mononucleotide P	P of phosphorylated carbohydrates	P of soluble polyphosphates	P of insoluble polyphosphates	RNA P	DNA P
P-enriched in absence of Ca^{++} (2.5 hours)	0.05	0.30	1.15	0.07	0.20	6.18	1.40
P-enriched in presence of Ca^{++} (2-3 hours)	0.19—0.23	1.11—1.37	2.91—3.18	0.54—0.78	1.98—2.23	6.34—6.80	1.38—1.42
Quantitative ratio of substances $\frac{\text{in presence of } Ca^{++}}{\text{in absence of } Ca^{++}} = K$	4.0	4.0	2.6	9.4	10.5	1.1	1.0

Table 3. The Phosphorus Compounds of Azotobacter vinelandii Growing on Phosphorus-Enriched Medium without Ca^{++} (2.5 Hours), and Then in the Presence of Ca^{++} (1 Hour)

Enrichment time in min	Acid-soluble fraction															P-enriched in absence of Ca^{++} (2.5 hrs)									Total nitrogen
	Orthophosphate			P of soluble polyphosphates			P of "ATP-ADP"			total mononucleotide P			DNA P			P-enriched in presence of Ca^{++} (2-3 hrs)			quantitative ratio of substances			"excess" P in RNA fraction			$\mu g-N$ / billion cells
	$\mu gP / 10^9$	spec act	counts *	$\mu gP / 10^9$	Spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	$\mu gP / 10^9$	spec act.	counts *	
Without Ca^{++} with P^{32}, 150 minutes	1.31	68.7	90.0	0.07	5.7	0.4	0.05	16.3	0.5	0.30	27.6	8.3	1.15	38.4	44.2	0.20	12.0	2.4	6.18	31.2	193.0	1.85	21.0	38.8	91.80
+Ca^{++} without P^{32} 20 minutes	1.43	36.4	52.0	0.15	10.1	1.5	0.10	28.1	2.8	0.43	24.0	10.3	1.26	18.1	22.8	0.62	46.8	29.0	7.20	2.70	194.0	2.10	18.6	39.0	92.00
40 "	1.51	18.1	27.3	0.26	15.3	4.0	0.15	30.8	4.6	0.50	21.6	10.3	1.50	16.5	24.7	0.87	36.8	32.0	7.46	25.3	189.0	2.33	16.1	37.5	92.10
60 "	1.70	7.2	12.2	0.42	20.0	8.4	0.18	29.0	5.2	0.80	14.8	11.8	1.73	13.9	24.0	1.35	28.0	37.8	7.61	24.2	184.0	2.50	15.5	38.8	91.97

* Counts per minute per billion cells.

The cessation of growth and nitrogen fixation in azotobacter grown on medium without calcium can be explained by impaired production of ATP, which is the universal energy donor.

This explanation also makes more understandable the retardation or absence of polyphosphate synthesis in azotobacter during calcium deficiency, since the production of polyphosphates occurs with the participation of ATP as the result of oxidative phosphorylation. The positions which we have brought forth are in agreement with the data in the literature (Schmidt, Hecht, and Thannhauser, 1946; Nickerson, 1949; Yoshida, 1952; Yoshida and Yamataka, 1953; Korchagin, 1954; Bukhovich and Belozerskii, 1958).

The experimental material given in Table 3 confirms the principal pathways of polyphosphate synthesis in the azotobacter cell which we discussed in detail in preceding works (Zaitseva, Belozerskii, and Novozhilova, 1960).

It is seen from the data in Table 3 that, during the enrichment of A. vinelandii cells in medium without calcium, radioactive orthophosphate may normally be incorporated into phosphorylated carbohydrates and, to a somewhat lesser extent, into the nucleoside monophosphates of the acid-soluble fraction. The relatively large incorporation of radiophosphorus into acid-soluble nucleoside monophosphates apparently explains the fact that the synthesis of nucleic acids is possible when there is a calcium deficiency of the medium.

Calcium deficiency of the medium strongly inhibited the incorporation of P^{32} into the labile phosphorus of the mononucleotide fraction, and especially into polyphosphates. When the azotobacter cells were transferred into medium containing calcium, the specific activity of orthophosphate immediately decreased sharply, while the activity of phosphorus in the "ATP – ADP" fraction and in polyphosphates increased simultaneously (Table 3).

The observed phenomenon is evidence of the fact that polyphosphate synthesis takes place with the participation of these very compounds.

On the basis of the analysis of the experimental data presented, it seems entirely probable to us that the effect of calcium on nitrogen and calcium metabolism is exerted via the oxidation–reduction energy conversions associated with respiration, with the active participation of ATP.

SUMMARY

The effect of calcium ion on nitrogen fixation is nonspecific, since its absence from the medium causes noticeable inhibition of the growth of A. vinelandii grown either on molecular or on ammonium nitrogen.

The absence of calcium ion in azotobacter affects the oxidation–reduction conversions of respiratory substrates which are coupled with phosphorylation. The disruption of specific links in the phosphorus metabolism of azotobacter when there is a deficiency of calcium in the medium causes reduced synthesis of ATP and polyphosphates. This, in turn, leads to cessation of growth of A. vinelandii.

During calcium deficiency, the amount of RNA synthesis decreases very little. The quantitative content of DNA in azotobacter cells does not depend on the calcium concentration of the medium. Thus, during the enrichment of cells with phosphorus (in the absence of calcium), the synthetic pathways of polyphosphates and of nucleic acids are not connected with one another.

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EXPERIMENTAL VARIABILITY IN ASPERGILLUS NIGER

II. CITRIC ACID PRODUCTION BY ASPERGILLUS VARIANTS OBTAINED BY ULTRAVIOLET IRRADIATION

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In the first report (Imshenetskii, Solntseva and Kuranova, 1959) a morphological description was given of four variants of Aspergillus niger 6/5, obtained by ultraviolet irradiation. As a result of repeated irradiation of conidia and selection of active cultures, the variants T-1 and T-2, which produce more citric acid than the original industrial strain, were obtained. A more detailed physiological description of the T-1 variant is given below. For comparison, the activity of the original A. niger 6/5 culture was studied at the same time.

EXPERIMENTAL METHODS

To obtain conidia the Aspergillus cultures were grown in tubes on wort agar containing 1.5% NaCl. After 4-day growth of the fungus on this medium at 32° an aqueous suspension of conidia containing 17–19 million per ml was prepared. About 0.5 ml of the aqueous suspension was seeded in 50-ml Erlenmeyer flasks containing 40 ml of medium of the following composition (in g): sucrose 5.0; NH_4Cl 0.35; $MgSO_4$ 0.05; KH_2PO_4 0.05; $ZnSO_4 \cdot 7H_2O$ 0.005; $FeSO_4$ 0.0025; tap water 100 ml. The pH of the medium was brought to 4.2 with hydrochloric acid. It was found during the work that seeding aqueous suspensions of conidia did not always produce solid, well-developed pellicles. Therefore, seedings of dry conidia were made subsequently on medium of the indicated composition. The inoculated flasks were kept at 32° for 36–42 hours, i.e., for the time necessary to produce fine white solid "corrugated" pellicles. Care should be taken that pellicle from the flask walls does not become detached. Such pellicles form little citric acid. Then the medium was removed from the flask and from under the pellicles, washed with sterile tap water, and introduced into 30 ml of medium composed of 20% sucrose solution containing 0.19% NH_4Cl. Flasks were again put in the thermostat at 32° and after definite time intervals the acid formed was determined in some of the flasks by titration with 0.1 N NaOH solution. Prior to titration the volume of medium was brought to the original volume with distilled water. Residual sugar according to Bertrand and dry weight of mycelium were determined at the same time. Calculation of the amount of sugar consumed and citric acid formed per g mycelium was made subsequently. In order to determine what acids are formed by the variant T-1, the amounts of citric, oxalic, and gluconic acids formed in the culture were determined. Acids were determined from the calcium salts.

DYNAMICS OF CITRIC ACID FORMATION

Experiments were conducted with cultures of A. niger 6/5 (original industrial strain) and mutant T-1 by the technique described above. In order to determine the dynamics of sugar consumption, citric acid production, and increase in mycelium weight, all analyses were carried out on the 3, 4, 5, 6, and 7th day. The results of these experiments are given in Table 1 and permit the following conclusions to be made:

1. Weight of T-1 mycelium is less than in the original strain.
2. Variant T-1 consumes more sugar per unit mycelium dry weight than the industrial culture.
3. The amount of citric acid in the variant T-1 culture is greater than in the original culture. Per unit mycelium dry weight, 46–84% more citric acid is formed.
4. Sugar consumption and citric acid production in variant T-1 are particularly active in cultures three and four days old.

Thus these data confirm the higher biochemical activity of the T-1 variant. It has increased oxidative capacity and produces more citric acid, both in the medium and per unit mycelium dry weight.

PRELIMINARY DATA ON CARBON BALANCE

An idea of the carbon balance should be obtained in making a comparison of the activity of the original aspergillus strain and the variant. In these experiments we did not attempt to identify all carbon-containing products, but restricted ourselves to determining the acids formed (according to citric), mycelium, and residual sugar; the remaining, unaccounted-for carbon belonged to carbon of carbon dioxide and unidentified products. The results of these experiments are given in Table 2. Citric acid carbon is considerably greater in T-1 than in the original culture, and sugar carbon remaining in the medium is less.

As for carbon of carbon dioxide, mycelium, and unidentified products, its amount is greater in strain 6/5.

Table 1. Dynamics of Citric Acid Production in Aspergillus niger 6/5 and Ultraviolet Mutant T-1

Index	Culture age, days	6/5	T_1	Index	Culture age, days	6/5	T_1
Citric acid produced in medium, %	3	7.0	8.1	Citric acid produced per g dry mycelium, g and %	3	4.8—100	7.6—159
	4	8.2	10.3		4	4.9—100	9.0—184
	5	10.2	12.1		5	5.8—100	9.4—162
	6	11.4	13.9		6	6.4—100	10.1—158
	7	13.0	15.0		7	6.9—100	10.1—146
Sugar consumed, %	3	13.82	14.03	Sugar consumed per g dry mucelium, g and %	3	9.5—100	13.2—139
	4	15.65	16.41		4	9.5—100	14.3—151
	5	17.71	17.67		5	10.2—100	13.7—134
	6	19.03	19.67		6	10.6—100	14.3—135
	7	20.52	20.19		7	10.8—100	13.6—126
Mycelium dry weight per 100 ml medium	3	1.462	1.064	Citric acid produced in % of sugar consumed	3	50.7	57.7
	4	1.650	1.151		4	52.4	62.8
	5	1.746	1.293		5	57.6	68.5
	6	1.781	1.372		6	59.9	70.7
	7	1.895	1.481		7	63.4	74.3

Table 2. Carbon Balance during Sucrose Oxidation by Aspergillus niger 6/5 and T-1 Variant

Culture	C of original sugar, g	C of remaining sugar, g	C of mycelium, g*	C of citric acid, g	C of CO_2 and other products, g
6/5	10.10	2.41	0.217	3.6	3.87
T_1	10.10	2.04	0.209	4.5	3.35

* The mycelium carbon is given only from a calculation made on the additional weight of pellicle in four days after change of medium.

Table 3. Citric Acid Production by 6/5 (Original Strain) and T-1 (UV Mutant) in Acute Experiments (18 Hours) (Average Figures)

Culture		Citric acid produced in % in medium	Pellicle dry wt. per 100 ml medium	Citric acid produced per g dry substance		Sugar consumed per g dry substance		Sugar consumed in % in medium	Citric acid produced in % of sugar consumed
				in g	in %	in g	in %		
6/5	1	4.3	0.507	8.5	100	21.9	100	11.1	38.6
	2	4.8	0.520	9.2	100	20.7	100	10.8	44.6
	Average	4.6	0.514	8.9	100	21.3	100	11.0	41.6
T_1	1	5.5	0.423	13.0	153	27.7	127	11.7	47.1
	2	5.5	0.320	14.1	153	30.8	149	9.9	55.8
	Average	5.5	0.372	13.6	153	29.3	138	10.8	51.5

Table 4. Acids Produced by T-1 Variant and Original Aspergillus 6/5 Culture (in %)

Acid in g per 100 ml med.	6/5				T_1			
	I	II	III	average	I	II	III	average
Citric	6.228	5.473	6.157	5.953	7.385	7.275	7.207	7.289
Gluconic	0.25	0.53	0.22	0.33	0.33	0.73	0.70	0.59
Oxalic	0.022	0.027	0.023	0.024	0.015	0.015	0.033	0.021
Citric acid in % of total acids	95.8	90.8	96.2	94.3	95.5	90.7	91.2	92.5

ACUTE EXPERIMENTS

The technique of these experiments differed from the regular ones in that only 5 ml of medium, instead of the usual 30 ml, was employed when replacing the medium under the pellicles with sucrose solution containing ammonium chloride. Duration of experiments was sharply curtailed, being 6 and 18 hours at 32°. The data from these experiments are given in Table 3.

The results of the acute experiments also indicate higher biochemical activity in the T-1 variant. The amount of citric acid it produces in 18 hours from the sugar consumed is 51.5%, while only 41.6% is produced by the original culture during the same time. Since the pellicle weight of the mutant is less, it produces considerably more (53%) citric acid per g of dry mycelium than the industrial strain. The fact is striking that the absolute amount of sugar consumed in the medium is the same for both strains.

NATURE OF ACIDS PRODUCED

The question naturally arose of whether the ratio between the acids produced had not changed in the mutant compared to the original culture. Experiments to answer this question were conducted by the regular technique. Duration of the experiment was four days. At the end of the experiment a quantitative determination was made in the culture fluid of citric, gluconic, and oxalic acids from the calcium salts. The data from these experiments are given in Table 4.

The gluconic acid formed by variant T-1 per 100 ml medium is 0.26 g higher, while the same amount of oxalic acid is produced by the two strains. Average citric acid content in culture fluid is higher in T-1, but owing to the somewhat higher yield of gluconic acid the percent citric acid of total acids found is a little less than in the original strain. There are essentially no appreciable changes in ratio of the acids produced in the T-1 variant. In the original strain the citric acid in percent of total acids is 94.3% and in the variant, 92.5%.

CITRIC ACID OXIDATION

The higher citric acid content in the experimentally obtained variant may be due to various causes. The suggestion was made in particular that the variant T-1 oxidizes the citric acid formed and retained in the medium less actively than the original culture.

The question of the possible oxidation of citric acid arose in connection with the results of experiments where mutants and original strain consumed all the sugar in the medium. Unequal consumption by the various strains of the citric acid formed could, of course, easily occur in these circumstances.

In order to check this possibility, 6% citric acid solution was added under the pellicles of four variants (2/4–10, T-1, and T-2) and the A. niger 6/5 industrial strain. Determinations were made after two and four days of the mycelium dry weight and citric acid by the method of Hartman and Hilling. The results of these experiments are given in Tables 5 and 6 and permit the following conclusions to be drawn:

1. Variants 2/4–10 and X oxidize citric acid with approximately the same activity as the original culture.
2. The oxidative capacity in variants T-1 and T-2 is greater than in the industrial culture. In four days the two cultures oxidize 22% more citric acid per g dry mycelium.
3. The higher citric acid content in variant T-1 cannot be due to the fact that it oxidizes the citric acid formed more slowly.

DISCUSSION OF RESULTS

The possibility of experimentally obtaining fungus variants that form more organic acids than the original form has been reported repeatedly in the literature. Kasatkina (1955) in our laboratory found that the wild form of Penicillium chrysogenum under laboratory conditions gives wrinkled variants that form considerably more gluconic acid than the original smooth form. Gardner, James and Rubbo (1956) were able to obtain from a wild strain of A. niger, by means of radiant

Table 5. Citric Acid Oxidation (5.95% according to Hartman) by Two-Day Pellicles of Aspergillus niger 6/5 (Original Culture) and Variants T-1 and T-2

Culture	After 2 days				After 4 days			
	mycelium dry wt., g	citric acid oxidized, g	citric acid oxidized per g dry myeclium		mycelium dry wt., g	citric acid oxidized, g	citric acid oxidized per g dry mycelium	
			in g	in %			in g	in %
6/5 (original)	0.2881	0.71	2.46	100	0.4423	1.41	3.19	100
T-1 (variant)	0.2205	0.75	3.40	138	0.3145	1.22	3.88	122
T-2 (variant)	0.3463	0.89	2.57	105	0.3921	1.52	3.88	122

Table 6. Citric Acid Oxidation (5.6% according to Hartman) by Two-Day Pellicles of Aspergillus niger 6/5 (Original Culture) and Variants 2/4–10 and X

Culture	After 2 days				After 4 days			
	mycelium dry wt., g	citric acid oxidized, g	citric acid oxidized per g dry mycelium		mycelium dry wt., g	citric acid oxidized, g	citric acid oxidized per g dry mycelium	
			in g	in %			in g	in %
6/5 (original)	0.4258	0.78	1.83	100	0.4481	1.23	2.75	100
2/4-10 (variant)	0.4056	0.70	1.73	95	0.4204	1.13	2.69	98
x (variant)	0.3799	0.69	1.82	99	0.4273	1.12	2.62	95

energy, variants forming more citric acid than the original wild culture. The amount of citric acid produced, however, was not great and such selection was of no practical importance. There are also studies in which the occurrence is described of Aspergillus mutants giving high yields of itaconic acid. Of importance in our work is the fact that the increase in citric acid production was achieved in an industrial strain of A. niger, employed over a long period in industrial production. Consequently, studies on experimental variability in fungi may result in raising the activity of cultured and selected strains. Of much theoretical interest is the physiology of the variants obtained, the T-1 culture in particular. It forms less mycelium and at the same time oxidizes sucrose more actively with formation of citric acid. As experiments in this paper have shown, this cannot be due to slower oxidation of the citric acid formed. On the contrary, the capacity to oxidize not only sucrose, but citric acid as well, is increased in the variant. Consequently, one may assume that it is the synthetic processes that have been hereditarily damaged by ultraviolet rays and that the enzyme systems associated with anabolism, with the formation of mycelium protein, have been deranged. At the same time the oxidative capacity has been increased, apparently, as well as the activity of the enzyme systems associated with phosphorylation and formation of organic phosphorus compounds in the mycelium. Further study of the physiology of variants will perhaps reveal their deficiency in certain amino acids or other compounds and if, by modifying the medium, it proves possible to increase fungus biomass while retaining the oxidative capacity per gram of mycelium at the previous level, the activity of citric acid production should increase still further.

The biomass decrease in the variants supports the general principle that the growth, cell division, and development are damaged earlier and more greatly by radiant energy than are the oxidative processes or the biosynthesis of such biologically active compounds as antibiotics or vitamins. It is to be anticipated that aspergillus mutants, which form more citric acid, will have more active respiration and will form more carbon dioxide during oxidation of various carbon-containing substrates than the original culture. One of the major problems in general microbiology is a thorough knowledge of the parameters of the process involved in obtaining variants of various microorganisms by means of radiant energy, regularly and reproducibly, that will more actively oxidize a variety of carbohydrates, alcohols, acids, hydrocarbons, and other compounds.

SUMMARY

1. Citric acid production was studied in Aspergillus niger 6/5 mutants, obtained by ultraviolet irradiation.

2. It was found from a study of the dynamics of sugar consumption, citric acid accumulation, and mycelium growth, that variant T-1, while oxidizing the same amount of sugar, produces more citric acid in the medium than the original industrial culture. Weight of mycelium formed in a growing variant T-1 culture is less than in the original strain; hence, this variant gives 46–84% more citric acid than the original strain when calculated per gram mycelium dry weight.

3. Carbon of mycelium, of liberated CO_2, and of unidentified products is somewhat greater in the original culture than in variant T-1. Citric acid carbon is greater and residual sugar carbon is less in the variant than in the original culture.

4. The increased oxidative capacity of the variant was confirmed in acute experiments on oxidation of sucrose to citric acid by the fungi. The citric acid yield in percent of sugar consumed in 18-hour experiments was 41.6% in the original culture, and 51.5% in the variant. Per gram dry mycelium, 53% more citric acid is formed in acute experiments.

5. The variant, as well as the original culture, produces citric acid principally (9.25%), and gluconic and oxalic acids in small amount.

6. The increased citric acid content in the variant cannot be due to slower oxidation of the citric acid formed. The variant oxidizes more citric acid per unit mycelium weight than the industrial strain on medium containing citric acid as sole carbon source.

7. The possibility is demonstrated of obtaining experimentally, by means of ultraviolet irradiation, mutants of aspergillus with greater oxidative capacity and greater citric acid production from the sugar consumed than the original strain.

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EFFECT OF ULTRAVIOLET IRRADIATION IN VARYING DOSAGE ON PRODUCTION OF ASPERGILLUS NIDULANS VARIANTS WITH ACTIVE PROTEASES

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We have pointed out in previous reports the possibility of producing by means of ultraviolet irradiation various mutants from mold fungi of Aspergillus genus, having increased proteolytic capacity (Brotskaya, 1954, 1956). In particular, strain n/7 was obtained from Aspergillus nidulans BIN 252, having proteolytic enzymes 2.5 times more active than the original form. However, these studies did not establish the effect of different doses of ultraviolet rays on the formation of morphologically modified variants with active proteases. It was also of interest to determine the stability of such variants.

It is well known that special attention must be given to variability in the cultures obtained in conducting work on selection. This is particularly true if the studies involve the use of various powerful agents. Even a single ultraviolet irradiation can alter the metabolism in microorganisms, which promotes the appearance of new strains, but the value of any new form will be determined primarily by its physiological and morphological stability. It is precisely these features that distinguish strain n/7, which has now retained its properties for more than 8 years under various conditions of maintenance in industrial production and in the laboratory. This culture itself was obtained "stepwise", i.e., by repeated irradiation and subsequent selection (Brotskaya, 1956). It was therefore of interest to determine whether it is possible to obtain stable, genetically modified, active variants by a single irradiation.

The purpose of the present study was to determine the effect of different doses of ultraviolet irradiation on the formation of mutants from A. nidulans with increased proteolytic activity, and to determine the stability of variants obtained after a single irradiation.

EXPERIMENTAL MATERIAL AND METHODS

A total of 275 different variants of Aspergillus nidulans, strain BIN 252, were studied.

L. A. Kuzyurina in our laboratory studied the stability of aspergillus conidia toward ultraviolet rays and variability in the mold fungi (Kuzyurina, 1959a, 1959b). A determination was made in these studies of the amount of energy in ergs applied to each irradiated fungal conidium.

Data on the doses employed are given in Table 1. We obtained 218 variants in which the proteolytic properties were studied.

Table 1. Exposures Employed (Distance of lamp from irradiated surface 50 cm)

Symbol for different doses	Duration of action	Energy in ergs per conidium ($\times 10^{-3}$)
A	10 sec	2.4
B	33 "	8.0
C	64 "	15.4
D	1 min 44 sec	25.0
E	3 " 20 "	48.0
F	6 " 40 "	96.0

The studies were conducted with 7-day cultures grown on wort agar at 28°. To determine proteolytic activity, they were then grown on bran extract which we employed in previous studies (Brotskaya, 1956). The medium was added in 40-ml amount to sterile 100-ml Erlenmeyer flasks and sterilized at 0.5 atm 30 minutes. Seed material was added on the basis of 400,000 conidia per milliliter of medium. Four flasks were inoculated in each variant of the experiment. The enzyme activity was determined in 2 flasks and the biomass dry weight in 2 others. The experiment was maintained at 28° for 5–6 days. The control was the original A. nidulans culture.

Proteolytic activity was determined by the following technique. The pellicle was ground carefully in a mortar and all the flask content was filtered through a paper filter. Protease activity was determined in the filtrate obtained. To 5 ml 2% casein* was added 5 ml distilled water, the mixture was heated to 37° and 2.5 ml of culture filtrate was added to it. The mixture was carefully shaken and kept in the thermostat at 37° for 2 hours. Then the degree of proteolysis was determined in the electric photocolorimeter with phenol reagent from the amount of tyrosine split off (Belozerskii and Proskuryakov, 1951).

How did the different doses of ultraviolet rays affect the enzyme activity of the cultures? Data on proteolytic

*2g finely pulverized casein was dissolved in 50 ml 0.5 Na_2CO_3 solution at 70°. After complete solution of the casein, 50 ml 0.01 N HCl was added for neutralization. The casein solution obtained had pH 6.75–6.60.

activity of the variants in relation to irradiation intensity are given in Figure 1. Proteolytic activity in the variants at first increases with increase in dose.

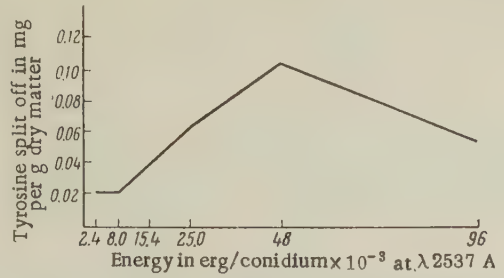

Fig. 1. Proteolytic activity of variants produced by various doses of UV rays.

This activity increases, however, only up to a certain point, and then a sharp drop in the curve sets in. Increasing the irradiation doses further does not result in obtaining more active strains. The most optimal, at wave length 2537 A, was dose E, equal to 48×10^{-3} erg/conidium (exposure time 3 minutes 20 seconds).

Of 218 cultures studied only 22 variants showed increased proteolytic activity (amount of tyrosine split off was 0.06–0.1 mg per gram of dry matter).

These variants belonged, morphologically, to the different types of mutants described by Kuzyurina (1959), and only one strain (E IV 10) was but little different from the original form. Data are given in Table 2 on enzyme activity of the cultures studied in absolute amount and in percent of total number of variants studied. Figure 2 is presented to give a more graphic conception of the number of variants with definite activity obtained at different doses. The original aspergillus strain (control) split off 0.01–0.02 mg tyrosine per gram of dry substance. The majority of variants produced at any of the exposures employed were of types with intermediate proteolytic activity. But with use of dose E not only were the most active mutants formed, but the total number was greatest. Consequently, a regular production of variants forming active proteases is possible when a certain irradiation dose is employed.

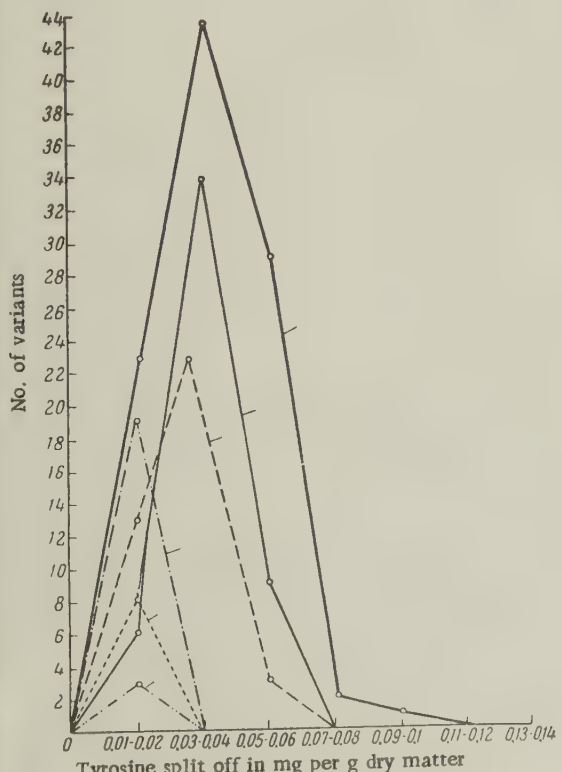

Fig. 2. Proteolytic activity of variants produced by various doses of UV irradiation.

In order to verify this assumption we repeated the experiments on producing proteolytically active cultures from the original A. nidulans strain, employing only dose E. As a result of these repeated tests 57 new, morphologically modified variants were obtained. Six cultures had increased proteolytic activity, of which 3 strains were analogous in enzyme properties to the 22 most active variants from the 218 cultures we studied.

Thus, the data obtained support our working hypothesis regarding the possibility of regularly producing proteolytically active strains from the original aspergillus strain studied. For the production of active aspergillus variants with other physiological properties, certain doses of ultraviolet irradiation will in all probability prove to be optimal.

STABILITY OF THE VARIANTS OBTAINED

As we have pointed out, all the cultures described above were obtained by a single irradiation. It is known, however, from the studies conducted in our laboratory that such strains very rapidly lose the newly acquired properties, even if transfers are not often made (Imshenetskii, 1951; Imshenetskii and Solntseva, 1960). In order to test the stability of the variants obtained, all 25 active cultures after passage 3 times on wort agar were kept 4 months under the following conditions. The conidia from 7-day well-developed cultures on wort agar were collected and kept in the dry state in sterile tubes, in sterile quartz sand, and in sterile soil. In addition, a culture on a wort-agar slant was covered with sterile vaseline oil. At the end of this period of storage, the cultures were transferred to wort agar and their enzyme activity determined. The results of these experiments are given in Table 3. As seen from this table, all the strains without exception had relatively rapidly lost the capacity to form active proteases and scarcely differed in their enzyme properties from the original culture. This fact serves, in our opinion, as one more demonstration that a single ultraviolet irradiation does not produce variants with stable properties. This is in agreement with data obtained by a number of other workers (Dulaney, 1953; Farrell, 1953; Ishihara, 1957). Only the "step-wise" action of so powerful an agent as ultraviolet radiation, combined with subsequent selection, can result in the production of stable, genetically fixed, useful forms.

Table 2. Proteolytic Activity of the 218 Variants Produced

Tyrosine split off in mg calculated per g dry matter *				
0.01—0.02	0.03—0.04	0.05—0.06	0.07—0.08	0.09—0.10
No. of strains				
73	101	41	2	1
In % of total no. of variants studied				
33.02	46.23	18.80	0.91	0.45

*Tyrosine split off in control was 0.01—0.02 mg.

Table 3. Effect of Storage on Proteolytic Activity of Aspergillus nidulans Obtained after a Single Ultraviolet Irradiaton at Various Doses

Culture no.	Initial activity, duration of expt. (days) 5		6		Activity after 4-month storage, duration of expt. (days) 5		6	
	dry wt. in g	tyrosine split off in mg per g dry mat	dry wt. in g	tyrosine split off in mg per g dry mat	dry wt. in g	tyrosine split off in mg per g dry mat	dry wt. in g	tyrosine split off in mg per g dry mat.
original	0.3647	0.027	0.4346	0.025	0.4459	0.024	0.4022	0.022
EIII 13	0.3621	0.055	0.3553	0.072	0.3048	0.031	0.3828	0.046
EIII 15	0.3984	0.059	0.4460	0.053	0.5267	0.046	0.5443	0.040
EII 5	0.3888	0.061	0.4567	0.055	0.5953	0.026	0.6603	0.023
EIV 5	0.6089	0.063	0.5777	0.065	0.7271	0.026	0.6885	0.027
EIV 2	0.3224	0.068	0.3964	0.059	0.7271	0.024	0.6512	0.032
EIV 7	0.3605	0.054	0.3936	0.050	0.6228	0.025	0.3504	0.024
EIV 9	0.3860	0.052	0.3054	0.045	0.6432	0.021	0.6457	0.028
EIV 13	0.4962	0.050	0.5523	0.046	0.6681	0.025	0.6069	0.031
EIV 10	0.2300	0.103	0.2704	0.083	0.3713	0.049	0.4260	0 043
EIV 18	0.3480	0.068	0.4137	0.061	0.4798	0.023	0.4957	0.023
EI 6	0.3219	0.062	0.4483	0.044	0.4319	0.044	0.4659	0.040
EI 7	0.2754	0.067	0.2356	0.078	0.5944	0.026	0.5320	0.038
EI 20	0.3041	0.064	0.3498	0.056	0.4098	0.034	0.5913	0.024
EI 21	0.2640	0.072	0.3598	0.059	0.6522	0.024	0.7233	0.028
EI 14	0.3127	0.052	0.4136	0.066	0.4811	0.021	0.5573	0.034
DI 7	0.3320	0.050	0.3640	0.062	0.7046	0.026	0.6382	0.028
DII 6	0.3312	0.050	0.3553	0.052	0.5134	0.033	0.5717	0.031
DI 19	0.3769	0.054	0.4122	0.053	0.5844	0.035	0.6061	0.034
EV 17	0.3351	0.058	0.4147	0.053	0.5891	0.037	0.5489	0.034
FII 10	0.3267	0.065	0.4931	0.037	0.5824	0.026	0.7695	0.024
FII 21	0.3319	0.061	0.3875	0.047	0.7381	0.025	0.6541	0.030
DI 4	0.2906	0.057	0.4328	0.048	0.3654	0.034	0.3320	0.020
1	0.2603	0.044	0.4436	0.050	0.3757	0.030	0.4700	0.029
8	0.4302	0.043	0.3569	0.052	0.3176	0.021	0.4032	0.033
55	0.3573	0.053	0.4416	0.045	0.3027	0.022	0.5433	0.032

SUMMARY

1. Proteolytic activity was determined in 275 variants obtained from the original Aspergillus nidulans BIN 252 culture by ultraviolet irradiation.

2. Increasing the irradiation dose increases the total number of variants with increased activity up to a certain limit. Further increase in dose does not contribute to the production of more active cultures, but, on the contrary, sharply reduces the number.

3. The optimal UV dose for producing the most active variants was found to be 48.0×10^{-3} erg/conidium (at wave length 2537 A). Under these conditions not only the largest number but also the greatest activity are noted.

4. A single exposure to UV rays does not result in the appearance of stable, genetically fixed, proteolytically active strains.

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RESPIRATION OF AN ASPERGILLUS NIGER MUTANT OBTAINED BY ULTRAVIOLET IRRADIATION

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Respiration, i.e., biological oxidation, is a most important function of the organism. Energy is liberated, during oxidative reactions, which is then utilized for a variety of synthetic processes by the growing and functioning cell.

Mutants obtained by ultraviolet irradiation of mold fungi differ from one another in quite a number of morphological characteristics.

Study of their respiration, however, has been very inadequate. Of particular interest is the oxidative capacity of mutants that actively produce citric acid. The present paper deals with this question.

EXPERIMENTAL METHODS

The work was carried out with the original Aspergillus niger 6/5 N, and a mutant obtained after 4 ultraviolet irradiations and designated T-1 (Imshenetskii, Solntseva, and Kuranova, 1959). This organism formed more citric acid than the original culture.

Cultures were kept in the icebox in tubes containing sterile soil or quartz sand. Prior to the experiment the cultures from soil or sand were seeded on wort agar from which, after 4–5 days, transfers were made to wort agar containing 1.5% NaCl, since active conidia formation was observed on saline agar. Cultivation was at 32°. Four-day cultures from the saline agar were used to inoculate experimental flasks. Conidia were washed from the slanted agar with sterile distilled water and filtered through a silk strainer. Filtered conidia were counted in a Goryaev chamber. Seedings were always made with a definite number of conidia.

Respiration was determined by three methods in order to obtain more reliable results.

In the first series of experiments, respiration was studied by the usual manometric method of determining oxygen consumption during respiration in the Warburg apparatus.

In these experiments the fungi were grown on a shaker revolving at 250 rpm. Synthetic medium A_4, suggested by Zhuravskii (1955) for submerged culture, of the following composition (in g) was employed: NH_4NO_3–2.5; $MgSO_4 \cdot 7H_2O$–0.25; KH_2PO_4–0.16; sucrose–10.0*; tap water to 1 liter; pH was brought to 4.5–5.0.

Medium was added to 250-ml amounts. The silk-filtered conidia were seeded in the amount two million conidia per ml medium. Duration of cultivation in most experiments was 20 hours. In order to decrease endogenous respiration, mycelium was centrifuged three times in phosphate buffer and then placed on a shaker in flasks containing a fresh portion of phosphate buffer for 5–6 hours.

The mycelium was then centrifuged and a thick suspension prepared from it in phosphate buffer (pH 5.91) such that 1 ml of the suspension was approximately equivalent to 10 mg biomass dry weight. To the main part of each vessel was added 1 ml of this suspension. Simultaneously, the same volume of suspension was placed on glass filters No. 1 to determine dry weight. Filters were dried to constant weight at 100–105°.

To the vessel side-arm was added 0.5 ml 0.5% glucose solution. To control vessels in which endogenous respiration of the mycelium was determined 0.5 ml of water instead of oxidation substrate was added. To the alkali well was added 0.25 ml 20% KOH. In experiments where the respiration coefficient was determined, 0.25 ml of water instead of alkali was placed in parallel vessels. In each variant of the experiment 2–3 duplicate manometers were used. Readings were taken every 30 minutes. Determination of Q_{O_2} was made from the data obtained – the number of microliters of oxygen consumed per hour per mg dry weight. Residual sugar in respiration vessels at the end of the experiments was determined by a micromethod.

In the second series of experiments, fungus respiration was studied by the method proposed by Ivanov and Shtern (1939). In view of the nature of the object, this method was somewhat modified.

The general form of the apparatus to determine fungus respiration is shown in Fig. 1.

The method is based on the principle of the binding by barium hydroxide of the carbon dioxide given off by the organism in a closed space. The apparatus consists of two flasks, one smaller one with a long neck bent at a right angle – the cultivator – and another larger one, a receptacle for storage of air with titrated barium hydroxide solution colored with phenolphthalein on the bottom. The color serves to judge the extent of barium hydroxide neutralization by the carbon dioxide given off during respiration. To avoid

*The amount of sugar in this medium was 15 times less than that given in G. I. Zhuravskii's formula, since the higher content of carbohydrates in the medium distorts the true values of the respiration from active oxidation of intracellular carbohydrates.

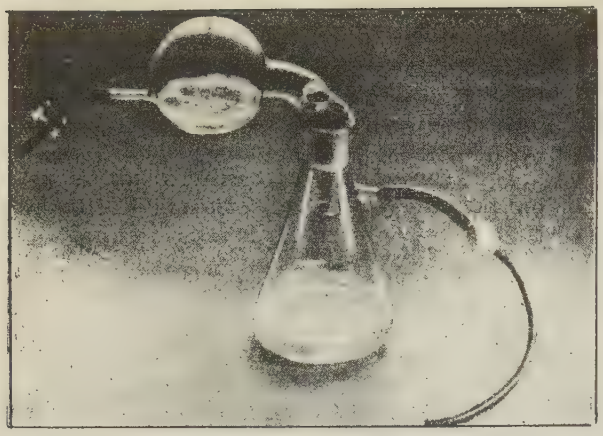

Fig. 1. Apparatus for studying fungus respiration.

entrance of atmospheric carbon dioxide, tubing with soda lime is connected to the receptacle outlet tubing. Volume of the cultivator is 0.5 liter; of the receptacle, 1.5 liters. To the cultivator is added 50 ml of medium of the following composition (%): sucrose 5.0; NH_4Cl—0.35–0.4; $MgSO_4$—0.05; KH_2PO_4—0.05; $ZnSO_4 \cdot 7H_2O$—0.005; $FeSO_4$—0.0025; tap water to 1 liter; pH is brought to 4.2.

Then the filtered conidia are seeded in the amount 200 thousand conidia per ml medium. Cultivator and receptacle are connected. At the juncture between their neck walls is packed sterile cotton, which is then saturated with a special hot grease that does not admit air and consists of two parts by weight of wax and one part of swine suet. This grease enables the flasks to be readily disconnected. After connecting the two flasks they are placed in the thermostat at 32°. From this moment the determination begins of the carbon dioxide given off by the fungus during respiration. In 42 hours, when a solid white pellicle has formed in the cultivator, the nutrient solution under it is replaced by 20% sucrose solution containing 0.19% NH_4Cl, on which the fungus is maintained 4–5 days.

In order that all the carbon dioxide given off by the fungus will be absorbed by the barium hydroxide, air freed of carbon dioxide by previous passage through a series of vessels containing 30% sodium hydroxide is passed through the whole system periodically during the experiment. The air enters through the side-arm of the cultivator. After definite time intervals receptacles containing fresh portions of barium hydroxide are substituted, and the barium hydroxide neutralized by carbon dioxide in the disconnected receptacles is titrated with 0.1 N HCl. The amount of CO_2 given off is determined from the difference in barium hydroxide titrations before and after the experiment.

In order to determine the amount of carbon dioxide present in flasks prior to beginning the experiment, analogous control flasks but without fungus are set up. In calculating the amount of carbon dioxide given off, this control correction is applied.

Residual sugar according to Bertrand, acid production by titration with 0.1 N NaOH, and biomass were determined in addition to carbon dioxide in these experiments. Fungus pellicles were dried to constant weight at 95–105°.

The third method employed in the study to determine respiration activity in surface cultures was based on the quantitative determination of oxygen uptake, developed for A. niger by Zhuravskii (1936) and subsequently modified by Kitavin (1952). The apparatus was set up according to the scheme proposed by Kitavin, but differing in that the volume of the oxygen receivers was 300 ml rather than 100 ml. Seedings were made with dry conidia. Potassium hydroxide for CO_2 absorption was added 24 hours after seeding, since otherwise the germination of conidia would have been extremely delayed. The apparatus and technique of determining oxygen uptake and carbon dioxide given off will not be described, since they were taken from the paper indicated, without modification.

Respiration Activity in Submerged Culture of Original Aspergillus niger 6/5 N Culture and Mutant T-1.

A comparative study was made of respiration in the original A. niger 6/5 N culture and in mutant T-1. Young 20-hour cultures of the fungi were employed first in determining respiration by the manometric method in the Warburg apparatus.

The most typical experimental results are given in Table 1, where the average data of seven analogous experiments are shown (with duplicate manometers in each).

On analyzing these figures it may be said that the respiration activity of the experimentally produced T-1 strain was always higher than in the original 6/5 N culture. The endogenous respiration was on the average 11–22% higher in mutant T-1 than in the

Table 1. Respiration Determined in Warburg Apparatus of Original Aspergillus niger 6/5 N Culture and Mutant T-1

Culture	Duration of expt. (min.)	O_2 taken up (μl)		CO_2 given off (μl)		Respiratory coefficient $CO_2:O_2$		Glucose consumed (mg per mg dry wt.)
		endogenous resp.	on glucose	endogenous resp	on glucose	endogenous resp.	on glucose	
original	60	9.0	29.5	9.0	29.0	1.0	0.98	—
mutant T-1		10.5	37.0	9.5	38.0	0.94	1.03	—
original		23.0	61.5	22.0	64.0	0.95	1.04	—
mutant T-1	120	26.5	76.0	25.0	78.0	0.94	1.02	—
original		33.5	80.0	32.5	79.5	0.97	0.99	—
mutant T-1	180	38.5	96.5	38.5	99.0	1.0	1.02	—
original		38.5	91.0	37.5	90.0	0.97	0.98	14.4
mutant T-1	240	46.5	109.5	45.5	111.0	0.97	1.01	17.3

Note: - signifies no determination.

original 6/5 N form. The energy of respiration of mutant T-1 in the presence of glucose was 18–28% greater on the average than that in the original 6/5 N culture. Glucose consumption in the 3–4-hour experiments in the Warburg apparatus was 16–22% greater in mutant T-1 than in the original 6/5 N culture. The respiration coefficient was everywhere close to unity.

The experiments with older mycelium, at different growth periods, revealed the same tendency (Table 2). Both endogenous respiration and respiration in the presence of glucose was 16–20% higher in mutant T-1 than in the original 6/5 N culture. It should be noted here that respiration in the two cultures decreased very insignificantly in the course of three days.

The results given above were obtained in submerged culture of the two strains. There are references, however, to the fact that submerged mycelium may differ in metabolism from surface mycelium (Umbreit, et al., 1951). It should be recalled that the A. niger 6/5 N culture is employed for industrial production of citric acid by the surface method. It has been noted a number of times that the best industrial surface strains have lower acid-producing capacity in submerged culture than the best submerged strains (Zhuravskii, 1955; others). This necessitated a study of respiration in these strains under the conditions natural to them, i.e., in surface growth.

Oxidation Capacity in Surface Culture of Original Aspergillus niger 6/5 N Culture and Mutant T-1.

It proved to be impossible to employ the manometric technique to study the physiology of respiration of fungi in surface culture, since even the same weights of the two cultures were not physiologically equivalent because of large differences in the degree of folding in the individual pellicle sections. Hence the method proposed by Ivanov and Shtern (1939) to study microbial respiration was used, a detailed description of which is given above. Duration of cultivation was 42–48 hours (without replacing solutions). These experiments showed that about the same amount of carbon dioxide was given off and bound by barium hydroxide in the original 6/5 N culture and in mutant T-1, and was even less in the mutant in some cases (Table 3). However, when the CO_2 given off in respiration was calculated per unit pellicle dry weight, the respiration activity in mutant T-1 was 20–23% higher than in the original culture inasmuch as the mutant biomass is 20–25% less than in the original culture.

These results do not give a full conception of the processes leading to citric acid production, since the latter is usually conducted on sugar solution (15–20%). Therefore, the next experiments were set up with the replacement of the nutrient medium after 42 hours with 20% sucrose solution containing 0.19% NH_4Cl. Determinations were made from the moment of replacing the medium at definite time intervals in the course of five days of the CO_2 given off, total acidity, residual sugar, and pellicle dry weight. In order to prevent the CO_2 given off from accumulating under the pellicle, air freed of CO_2 was passed periodically through the vessels.

Table 2. Oxygen Uptake (μl/mg/hr) in Relation to Culture Age in Original Aspergillus niger 6/5 N Culture and Mutant T-1 (Average data from three experiments with duplicate vessels)

Culture	Endogenous resp.			Resp. on glucose		
	days of growth			days of growth		
	1	2	3	1	2	3
Original	41.1	40.5	35.7	106.8	98.9	81.4
Mutant T-1	48.3	47.1	41.8	122.7	118.7	95.2
Respiration increase in mutant over original form (%)	19	16	17	18	20	17

Table 3. Energy of Respiration in Surface Growth of Original Aspergillus niger 6/5 N Culture and Mutant T-1 (Average figures from duplicate flasks)

Culture	Expt. no.	Biomass dry wt. (g)	CO_2 given off		
			ml	mg	mg per g dry wt.
Original	1	0.7762	410.1	0.902	1162
	2	0.6532	455.2	1.001	1532
	3	0.6831	428.8	0.943	1380
	4	0.8868	452.3	1.000	1240
Average		0.7498	436.6	0.960	1281
Mutant T-1	1	0.5873	390.6	0.859	1462
	2	0.4777	449.2	0.988	2070
	3	0.5546	431.3	0.949	1729
	4	0.7903	455.1	1.001	1267
Average		0.6025	431.5	0.949	1575

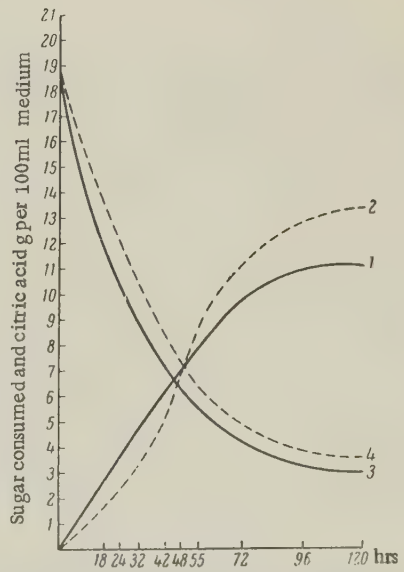

Fig. 2. Sugar consumption and citric acid production in original Aspergillus niger 6/5 N culture and mutant T-1. 1) Citric acid produced by original 6/5 N culture; 2) citric acid produced by mutant T-1; 3) residual sugar in original 6/5 N culture; 4) residual sugar in mutant T-1.

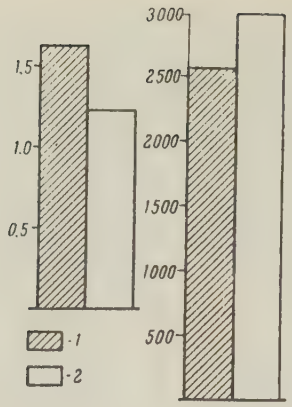

Fig. 3

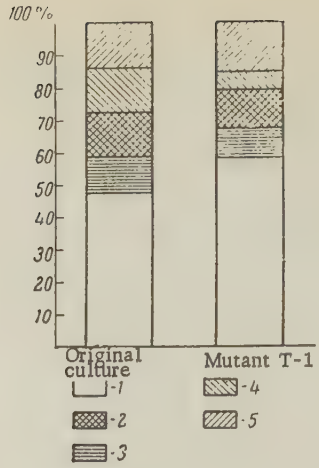

Fig. 4

Fig. 5

Fig. 3. Biomass dry weight in g. 1) Original culture; 2) mutant T-1.

Fig. 4. CO_2 given off in mg per g pellicle dry weight. 1) Original culture; 2) mutant T-1.

Fig. 5. Carbon balance in Aspergillus niger 6/5 N and mutant T-1 (in % of carbon of given sugar). 1) Citric acid carbon; 2) CO_2 carbon; 3) biomass increment carbon; 4) carbon of unidentified products; 5) residual sugar carbon.

Table 4. Respiration with Continuous Access of Oxygen in Original Aspergillus niger 6/5 N Culture and Mutant T-1
(Average figures of 2 experiments with duplicate flasks)

Culture	Duration of expt. (hrs.)	Biomass dry wt. (g)	Acidity (ml 0.1 N NaOH)	CO_2 given off			O_2 uptake			Respiratory coefficient CO_2: O_2
				ml	mg	mg per g dry wt	ml	mg	mg per g dry wt.	
Original	48	0.711	9.9	902	1984	2790	1130	1605	2243	0.79
Mutant T-1		0.521	11.2	884	1945	3733	1110	1576	3024	0.79
Original	96	0.996	15.4	651	1432	1437	810	1074	1074	0.80
Mutant T-1		0.782	18.6	664	1461	1882	830	1179	1507	0.80

In analyzing these experiments (Figs. 2–4)*, a considerable increase can be seen in the carbonic acid given off by both cultures in sugar solution compared to that of the basic nutrient medium containing 5% sugar (compare Table 3). Here the respiration in mutant T-1 is almost 14% greater than in the original culture (Fig. 4).

Since mutant T-1 growth lags somewhat behind the original culture at first, its active acid-production period begins later (Fig. 2); by the end of the experiment, however, mutant T-1 produces 24.6% more citric acid (in % of sugar consumed) than the original 6/5 N strain, and 55% more when calculated per unit mycelium dry weight. Here the amount of sugar consumed (in %) is about the same in the two cultures. However, the mutant biomass is 75% that of the original culture at the end of the experiment (Fig. 3).

Since the mutant utilizes about the same amount of sugar as the original culture and produces more citric acid with lower pellicle weight, the enzymes that form citric acid from sugar can be assumed to be more active. Consequently, sugar is utilized by mutant T-1 chiefly to synthesize citric acid.

This is confirmed by the carbon balance diagram (Fig. 5) calculated from these experiments and showing that mutant T-1 expends more sugar carbon for citric acid production than does the original 6/5 N strain.

Inasmuch as a large difference in the amount of citric acid is observed between the two cultures while other amounts of carbon — of biomass, CO_2, and residual sugar — are about the same, a large difference is also observed in the unidentified products.

If it be assumed that irradiation produced activation in the mutant of the enzyme systems responsible for citric acid formation, then perhaps the lower amount of unidentified products in T-1 is due to the fact that these "activated" enzymes oxidize sugar more completely to citric acid while the original culture does not decompose all the sugar utilized to citric acid, but forms a certain quantity of less oxidized compounds that fall in the category of "unidentified prod-

*For brevity the results of determinations made in phase II are given in Figs. 2-4, i.e., after replacing nutrient medium with sucrose solution. Pellicle dry weight was determined at the time of replacement of medium in order to calculate biomass increase in sugar solution at the end of the experiment.

ucts"; these might perhaps be mannite, glycerol, polysaccharides, and such products as observed in Penicillium chrysogenum by Raistrick, et al. (1931).

Two more experiments in addition to these were conducted on the respiration of surface cultures in Kitavin's apparatus. Oxygen uptake, carbon dioxide given off, biomass, and acidity were determined. In Table 4 are given the results obtained after replacement of the basic medium with sucrose solution.

The difference in energy of respiration in the original culture and in mutant T-1 was revealed even more distinctly in these experiments. Thus, the carbon dioxide given off and oxygen taken up was 30–38% greater in the mutant than in the original culture when calculated per g dry weight.

In conclusion, I acknowledge deep gratitude to Professor A. A. Imshenetskii for valuable advice and guidance of this work.

SUMMARY

1. The physiology of respiration was studied in the original Aspergillus niger 6/5 N culture and the mutant T-1, obtained by 4-fold ultraviolet irradiation of conidia of the original culture.

2. It was found that mutant T-1 has higher respiratory activity than the original culture, regardless of the method of determining fungal respiration.

3. Respiration of mutant T-1 was 18–28% higher than in the original culture, when determined in submerged culture in the Warburg apparatus.

4. Respiration of mutant T-1 was 20–23% higher than in the original culture in surface culture without replacement of solution in the apparatus of Ivanov and Shtern, and 14% higher with solution replacement.

5. Respiration of mutant T-1 was 30–38% higher than in the original culture in surface culture with continuous access of oxygen in Kitavin's apparatus.

6. Mutant T-1 has a higher acid-production capacity than the original culture, as well as increased respiratory activity.

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ANAEROBIC PROACTINOMYCETES

II: MORPHOLOGY

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The information in the literature on the morphology of anaerobic proactinomycetes is not extensive and often contradictory. Very few studies deal with the comparative description of the considerable number of strains of organisms referred to as the anaerobic proactinomycetes and of biologically related organisms. In view of this, the purpose of the present study was an attempt to provide such a description. The study was made with anaerobic proactinomycete strains isolated from various natural sources. The criteria employed for classifying organisms in the anaerobic proactinomycete group are set forth in a previous paper (Kalakutskii, 1960).

METHODS

Organisms. The organisms employed are listed in Table 1.

Medium. Two media were used: meat-peptone agar containing 0.5% glucose, and starch-yeast medium. Its composition: 10% yeast autolyzate 50 ml; soluble starch 10 g; K_2HPO_4 1.0 g; $MgSO_4$ 0.5 g; NaCl 1.0 g; $CaCO_3$ 1.0 g; tap water 1 liter. Growth of all strains was appreciably less on this medium than on meat-peptone medium. This refers particularly to strains of Actinobacterium meyeri and Actinobacterium israeli.

Conditions of anaerobiosis. See previous paper (Kalakutskii, 1960).

Culture of microorganisms on membrane filters. A sterile membrane was applied to the surface of agar medium. The filter surface was inoculated with a suspension in sterile tap water of cells from an 8–10-day culture on MPA containing glucose. After 1, 2, 3, 5, and 7 days of incubation the filters were removed, kept one hour in formalin vapor, air-dried, sectioned, and mounted in balsam. Preparations were examined with the phase-contrast system. Filters were in some cases stained with erythrosin, or treated with 10% aqueous tannin solution 30 minutes, rinsed, and stained with 0.05% safranin.

Staining of chromatin material was done in offprint preparations fixed in formalin vapor. Preliminary experiments with Carnoy's fixative showed that it has no particular advantage in this case. Staining was done by the following methods:

1. According to Pekarskii with counterstaining of cell membranes (Peshkov, 1955) with tannic fuchsin. Hydrolysis in 1 N HCl at 58–60° seven minutes was employed. In a modification of this method, cells were treated with ribonuclease instead of hydrochloric acid hydrolysis. To a suspension in distilled water of cells

Table 1. List of Cultures Employed

Nos.	Where isolated	Conditions under which isolated
101, 102, 103, 104, 106, 108	Pyatigorsk, Lermontov No. 1 spring	Anaerobic
109	2nd drilling, Baku, Kalinovskoe deposit	"
124	Lermontov No. 2 spring, Pyatigorsk	"
128, 130, 131, 132B	Narodnyi spring, Pyatigorsk	"
145, 149, 153, 155, 158	Water in subway tunnel, Moscow	"
169, 170, 171	Black Sea, 500 m depth	"
182, 183, 185	Drilling No. 32, health resort Goryachii Klyuch, Krasnodar krai	"
178	From tooth film	"
233, 237	" " "	Aerobic
172, 175, C-3, C-4, C-6, C-7, C-9	Air in buildings	Anaerobic
151	Pyatigorsk, Naklonnyi spring	Aerobic
255	" " "	Anaerobic
P-1, P-6, P-7, P-8, 260	Lermontov No. 1	"
257, 258	Essentuki, drilling No. 2	"
249	Drilling, Baku	"
303	Garden soil*	"
306, 307	Chernozem soil from Armenia	"
Actinobacterium israeli 1641A	Museum of Pasteur Institute	"
Actinobacterium meyeri 1371C	" " " "	"

*For isolation of anaerobic proactinomycetes from soil, the specimens were spread in petri dishes, moistened thoroughly, and covered with glass under which filter paper was placed. After 2–3 weeks of incubation at 37° isolations were made from the areas colored dark by sulfides.

killed by 3–5-minute boiling crystalline ribonuclease (L. Light & Co., Ltd., England) was added to final concentration 1 mg/ml. It was maintained 20 hours at 50–52°. Then the preparation was made and stained with Giemsa stain and treated subsequently according to the procedure indicated above.

2. Staining with iron hematoxylin according to Heidenhain (Omelyanskii, 1940) was conducted with mordanting in 2.5% alum solution for 24 hours, actual staining for 24 hours, and differentiation 10–25 minutes.

3. Staining with methyl green-safranin according to Prazhmovskii was done in accordance with instructions in Omelyanskii's handbook.

4. All the methods enumerated were employed jointly, not less than two being used in each individual case, and Pekarskii's method with counterstaining according to Peshkov was used in all cases.

Staining for spores was carried out by the method of Moeller (Omelyanskii, 1940) with Ziehl's fuchsin. The method was also employed of fluorochroming preparations with brilliant sulfoflavine, counterstaining with korifosfin and then examining in ultraviolet light, as is recommended for detecting bacterial spores by G. A. Medvedeva in the laboratory of Professor M. N. Meisel'.

Staining cell membranes was carried out by the method of Gutstein (Peshkov, 1955) or the method of Hale (1957) with aqueous methyl green solution.

Gram staining was done in accordance with the method described in Omelyanskii's handbook.

RESULTS AND DISCUSSION

It is impossible in the present report to present all the material on all the cultures employed in the study. Hence, in the ensuing presentation, the material on certain cultures has been given in more detail, while others are merely mentioned in order to confirm basic principles demonstrated in the former.

General Description of Culture Morphology.

All the culture studied have the following general morphological features: 1) formation in the early stage of forms characterized by rudimentary mycelium; 2) cells nonmotile and gram-positive. These features enable us to classify with assurance these organisms under the actinomycetales. The first characteristic appears to be quantitatively different in different cultures; they form a chain of gradually more complex forms in this respect. Regarding the structure and the development of anaerobic members of the ray fungi, this is described variously by different authors. Thus, Erikson (1940) speaks of the true mycelium they form, Rosebury et al. (1944) describe them as diphtheroid forms, while Beerens (1953) mentions rods and diphtheroids. We have endeavored in our work to adhere to the rule—study culture morphology according to growth stages. Experience has demonstrated that nonobservance of this rule and comparison of cultures of different ages results in confusion.

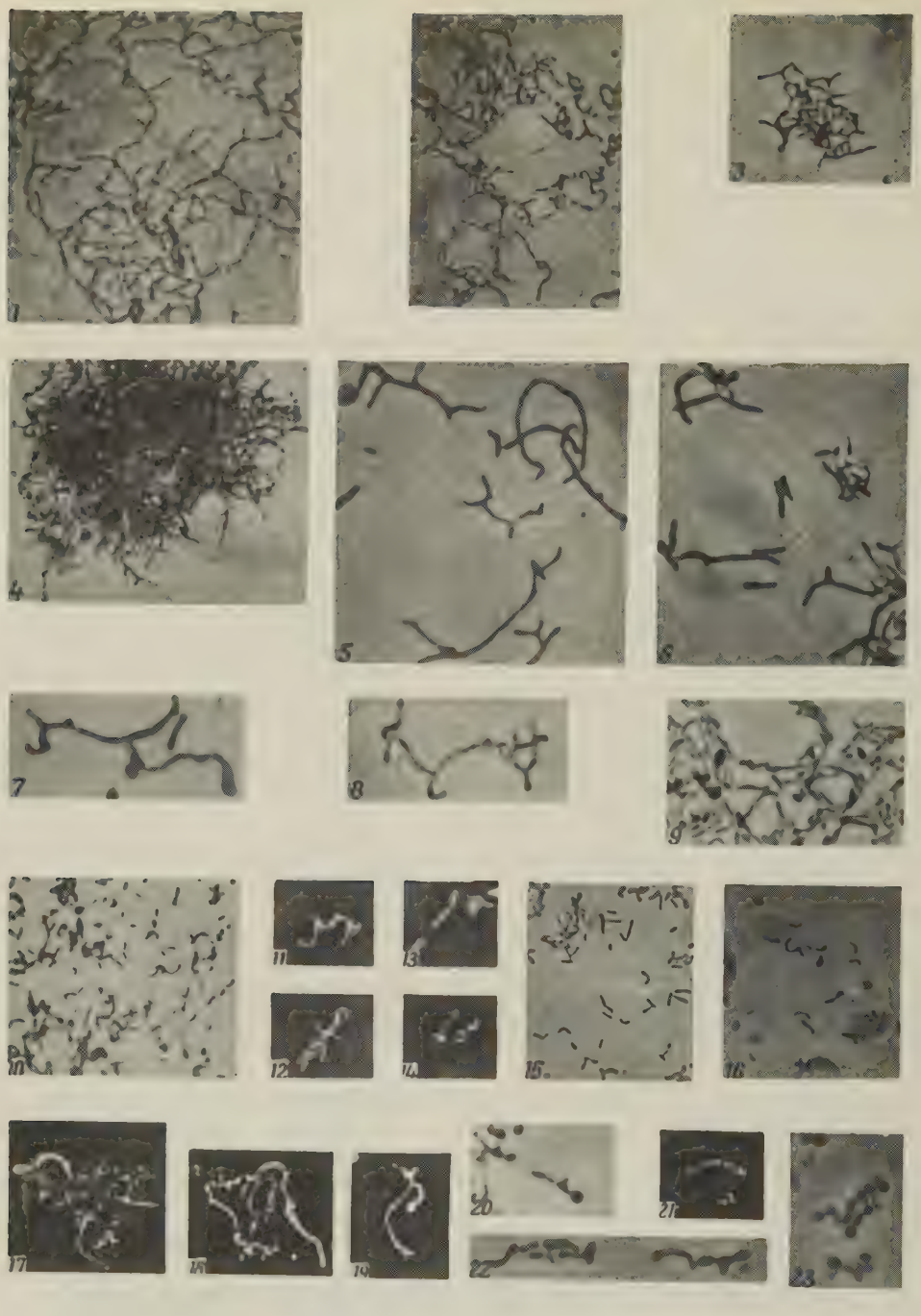
Morphology of Young Colonies.

The study of the morphology of young 1–2-day colonies was found to give a completer conception of the growth cycle in the given organisms. Usually at 18–20 hours after inoculating a filter with a suspension of an old culture, containing chiefly rod-shaped and rounded cells, the growth of young filaments of mycelium can be seen. Although the very term "mycelium" is understood differently by different authors, it is nevertheless sufficiently typical to distinguish cultures with respect to it. Thus, A. meyeri forms in some cases long, profusely branching mycelium (Fig. 1) whose appearance in the first growth stage is hard to distinguish from that of more highly organized members of the ray fungi. Ability to form such mycelium was present in this culture only; we did not observe it in the other cultures studied. The same culture forms in some cases shorter, fragmenting mycelium (Fig. 2). Mycelium formed by the other cultures (Figs. 3, 4) is usually shorter. True branching occurred in all cases. The primary mycelium formed by the cultures studied can be compared to the mycelium observed also in the aerobic proactinomycete, nocardia (McClung, 1954). On the other hand, short mycelial formations are noted in certain mycobacteria (Krasil'nikov, 1938). It is known to be difficult in a number of cases to make a choice between proactinomycetes and mycobacterium in classifying an organism. We will note that certain authors (Kudoh, 1956) have recently denied the existence of true branching in mycobacteria.

The fact that rudimentary mycelium was formed in all the cultures studied even in the first days of growth raises doubt regarding the suggestion made by certain authors (Morris, 1951; Batty, 1958) concerning the existence of a special, complex growth cycle in the anaerobic proactinomycetes. In fact, according to the scheme of these authors, anaerobic proactinomycetes grow in the form of septate rods in the first few days of growth, and only form septate primary mycelium after quite a long period. The primary mycelium was not septate in any of our cultures, and simply begins to disintegrate rapidly with culture age. After 2–3 days it was impossible to observe the further growth of mycelium in the colonies, since they become nontransparent and structural details cannot be detected. Further observations were therefore conducted in offprint preparations. Here the disintegration of the primary mycelium elements into rod-shaped and coccus-shaped cells of irregular form is seen well. The primary mycelium is often so unstable that, even in offprint preparations of 1-day cultures, frequently only the rods into which the mycelium has disintegrated can be seen.

Morphology of Cells.

The primary mycelium in various cultures differs in stability. Many cells in offprint preparations from 1–2-day cultures have, as a rule, the shape of rods, Y- and V-elements, and sometimes cocci. These elements predominate in old cultures. A. meyeri had the most



Figs. 1–4 and 10–mag. 900×; Figs. 11, 12, 13, 14, 15, 16, 17, 18, 19, 20–mag. 1000×; Fig. 7–mag. 2000×. Others–1800×; Fig. 1–4 on filter. Fig. 5–31–offprint preparations.

Fig. 1. Actinobacterium meyeri. 1-day culture on filter on MPA with glucose. Phase contrast. Fig. 2. Same Fig. 3. Strain No. 101 under same conditions. Fig. 4. Strain No. 303 under same conditions.

Fig. 5. Actinobacterium meyeri. 1-day culture on MPA with glucose. Phase contrast.

Fig. 6. Same, 3-day culture. Fig. 7. Strain No. 101. 2-day culture on MPA with glucose. Phase contrast.

Fig. 8. Strain No. 101. 7-day culture on MPA with glucose. Staining of cell membranes according to Gutstein.

Fig. 9. Strain No. 7. 7-day culture on MPA with glucose. Phase contrast.

Fig. 10. Strain No. 108. 3-day culture on MPA with glucose. Gram stain.

Fig. 11. Strain No. 101. 3-day culture on MPA with glucose. Anoptral system according to Wilska-Reichert.

Fig. 12. Strain No. 103 under same conditions. Fig. 13. Strain No. 108 under same conditions.

Fig. 14. Strain No. 106 under same conditions.

Fig. 15. Actinobacterium israeli. 3-day culture on MPA with glucose. Stained according to Hale.

Fig. 16. Strain 103. 3-day culture on MPA with glucose. Stained according to Hale.

Fig. 17. Strain 101. 3-day culture on MPA with glucose. Anoptral system. Fig. 18. Same. Fig. 19. Same.

Fig. 20. Same. Fig. 21. Strain No. 101. 2-day culture on starch-yeast medium. Phase contrast.

Fig. 22. Same, 4-day culture. Fig. 23. Same, 7-day culture.

stable mycelium. Many mycelial fragments are seen (Fig. 5) in preparations of 1-day cultures. The number of fragments decreases with age (Fig. 6), and they become shorter. There are also many mycelial fragments in young cultures of strain 101 (Fig. 7). In older 7-day cultures the main bulk of cells are rods and Y-elements (Fig. 9). Even at this age some of the cells in this culture nevertheless retain true branching and the appearance of mycelial fragments (Fig. 8). The same type of formation and growth of mycelium was present in cultures Nos. 102, 158 and, in part, 169. The rudimentary mycelium in all the other cultures disintegrates even more readily. Many authors, particularly in the medical literature, employ gram-staining in describing the morphology of anaerobic members of the ray fungi. This method of staining, according to our data, cannot be conclusive in determining the cell morphology in anaerobic proactinomycetes. What appears to be branching (Fig. 10) in gram-stained preparations is actually not (Figs. 11, 12, 13, 14). The shape of the cells into which the primary mycelium usually disintegrates—rod-shaped, pear-shaped, coccus-shaped—can be seen in Figs. 11, 12, 13, 14, 15, and 16).

"Bulb" Formation.

It was believed that bulb-like cells or "bulbs" are formed by the pathogenic anaerobic proactinomycetes only when they are growing in infected tissue in nodes. There is but one reference (Bayne-Jones, 1925) in the literature available to us that "bulbs" may be formed in artificial culture on medium without serum.

A number of our strains formed "bulbs" in culture; however, the number was not great, they were small in size, and it was often difficult to distinguish them from ordinary slightly inflated cells. Formation was of a sporadic type. In contrast to this, cultures Nos. 101 and 102 formed "bulbs" of typical structure (Figs. 17, 18, 19, 20) with great regularity. The "bulbs" could be observed both in very young (1-day) cultures and in old (10–20-day) ones, on agar and in liquid media. The diameter of the "bulbs", as seen in the photographs (Figs. 17, 18, 19), may exceed that of the average cell (0.6–0.9 μ) 4–5 and more times. Inside these "bulbs" in some cases (Fig. 20) there can be discerned 1–3 rounded bodies (these areas are often stained with nuclear stains). The regularity of the formation on different media and the typical structure of the "bulbs" formed by cultures 101 and 102 suggest that this characteristic is in the given case a stable morphological species characteristic.

Effect of Medium Composition on Cell Morphology.

The starch-yeast medium used in the study was obviously far from optimal, since it favored the appearance of extensive cell polymorphism. The main features of this were: formation of inflated cells, formation of spherical cells with nontransparent contents (Figs. 21, 22, 23).

Presence of spores could not be demonstrated either by following culture growth stages or by various staining methods. It may be suggested that the workers who describe spore formation in anaerobic proactinomycetes (Bisset, 1957) were dealing with small "bulbs" in the cultures or with pleomorphic cells due to the medium.

Change in Nuclear Material with Culture Age.

Although no staining agents exist at present so specific for bacterial nuclear material, with use of simple methods of treatment, as to permit a final judgement to be made, yet by the joint use of a number of methods assumptions can be made with a certain assurance. The changes in cell nuclear material according to growth stages were very similar in all the cultures studied. In the early growth stages (1–2 days on optimal medium—MPA with glucose) the cell protoplasm in anaerobic proactinomycetes is highly basophilic. Neither acid hydrolysis nor treatment with tannin or ribonuclease can overcome this basophilia and reveal differentiated nuclear structures (Figure 24). By four days of growth the chromatin areas can already be differentiated from lighter areas of the protoplasm (Figs. 25, 26). By seven days of growth the differentiation in protoplasm has become especially clear (Figs. 27, 28, 29, 30). The cell at this time usually contains 1–3 (occasionally more) chromatin granules. Some are in the center, others in a terminal position. Chromatin granules are not infrequently encountered in "bulbs", when the latter are formed. Small chromatin bodies can often be seen at the site of cell branching. The chromatin bodies are usually rounded in shape and occupy all or almost all the cell width. Sometimes they are of unequal size. In a number of cases they are arranged along the cell, occupying areas corresponding to 2, 3, or more individual smaller bodies. Growing of cells on unfavorable (starch-yeast) medium exerts a very strong effect on the appearance of chromatin material. Even at 2 days of growth (Fig. 31) the protoplasm here is so far differentiated that the chromatin bodies are readily observed, 2–3 in a cell. It is difficult to draw any final conclusions regarding the role in the cell of the chromatin bodies observed. In comparing the cycle of changes in nuclear material of anaerobic proactinomycetes and of actinomycetes (Prokof'eva-Bel'govskaya et al., 1958), their great similarity may be noted.

General Description of Growth Cycle.

The cells after seeding germinate and produce a primary mycelium. The latter is well-marked in some cultures (A. meyeri), less so in others (Nos. 101, 102, 158, 169, 307, and others), and still less in a third group (Nos. 303, -6, -8, 103, 106, 108, 104, and others). In addition to length the mycelium differs in stability. In some cultures (A. meyeri, 101, 102) it is stabler and in others (A. israeli, 158, 169, 307, and others) less so. Young cells are basophilic and stain homogeneously with nuclear stains. After one day of growth and in parallel with further growth the primary mycelium begins to fragment into Y- and V-elements, rods, and cocci. These elements determine the culture morphology for the duration of most of the growth span. The cell contents differentiate as the cell grows; chromatin material appears in the areas stained with nuclear stains. The growth cycle as a whole is similar

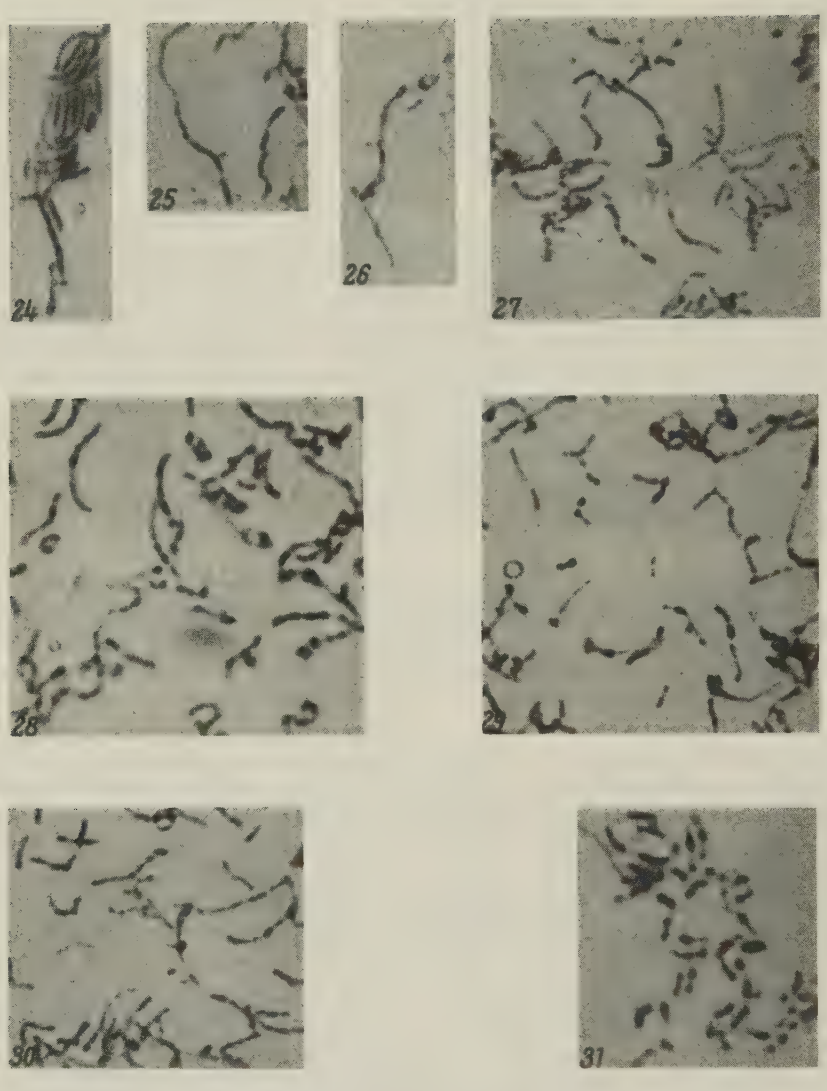


Fig. 24. Actinobacterium meyeri. 2-day culture on MPA wigh glucose. Pekarskii stain and Peshkov counterstain.
Fig. 25. Actinobacterium meyeri. 4-day culture on MPA with glucose. Hematoxylin stain with previous ribonuclease treatment.
Fig. 26. Actinobacterium meyeri. 4-day culture on MPA with glucose. Hematoxylin stain with previous ribonuclease treatment.
Fig. 27. Same, hematoxylin stain.
Fig. 28. Strain 101. 7-day culture on MPA with glucose. Pekarskii stain with Peshkov counterstain.
Fig. 29. Same, Prazhmovskii stain. Fig. 30. Same.
Fig. 31. Strain 101. 2-day culture on starch-yeast medium. Pekarskii stain with Peshkov counterstain.

in the anaerobic proactinomycetes to that in the aerobic proactinomycetes and mycobacteria (Krasil'nikov, 1934, 1938). Great fragility and poor development of the primary mycelium is again typical.

The study was made under the guidance of Professor N. A. Krasil'nikov, to whom the author expresses deep appreciation.

SUMMARY

The morphological features in different stages of growth were studied in 48 cultures isolated from various natural sources and considered to be representative of the anaerobic proactinomycetes. In the early growth stages all cultures form a primary mycelium that is very unstable and rapidly disintegrates into individual rod-, pear-, and coccus-shaped cells. Cell protoplasm, basophilic in early growth stages, partially loses this property with age; chromatin bodies become visible in the cells, usually 1–3 per cell. The culture medium exerts considerable effect on cell morphology and protoplasm basophilia. Spores are not formed by anaerobic proactinomycetes.

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ON THE PROBLEM OF CLASSIFICATION OF YEASTS

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The monographs on classification by the Dutch school, and that by V. I. Kudryavtsev, have opened a new era in the taxonomy of yeasts, thanks to the summarizing of results from investigations made over the past twenty years. Numerous scientists are now working in this special field of knowledge on the basis of these investigations. These studies require a broad knowledge of the yeast flora of the earth and promise to give results of value to the fermentation and food industries. Various problems in conducting these studies have been brought up; acquaintance with them of large numbers of microbiologists should assist in developing this branch of science. This had induced me to record my observations, as have other authors (Kudryavtsev, 1955; Barnett, 1957; Phaff, 1956).

Present-day taxonomy proceeds from two fundamental observations:

1. Among the many millions of living creatures inhabiting the earth are noted distinct groups comprising organisms similar in many properties. Such groups are termed taxonomic groups, or taxons (species, genera, families, etc.). Man learned long ago how to distinguish injurious and useful species. As for defining the boundaries between individual species, this has always depended on the subjective judgement of the worker.

2. Previously existing and present-day creatures are bound into a single whole by the process of evolution. Although there is disagreement in explaining the pathways and causes of evolution, the existence of evolution is no longer disputed by anyone.

The taxonomic investigation of yeasts is also based on these generally recognized concepts. The defining of species boundaries, however, is associated with serious difficulties.

Problem of Describing Species.

The methods of describing species were first worked out on the higher organisms. In the later study of yeasts and microorganisms in general they were subjected to substantial modification.

Higher organisms are described from specimens collected in the world of nature. Such specimens are termed semaphoronts in the zoological literature (Hennig, 1950). Nature does not provide the microbiologist with semaphoronts. He deals with pure cultures of microbes and these are the object of observation.

We know from experience the importance of the cultural conditions in affecting the results: glassware, nutrient medium, temperature, etc. The cultural conditions are set up by the investigator and the properties inherent in the given organism are studied under these conditions. This is possible only when appropriate experiments are set up to reveal each of the properties. The number of possible experiments may be large. Certain of them are greatly preferred by workers in yeast classification, and they group their cultures into species on the basis of the results obtained.

By employing different experiments we might arrive at a different grouping. The basic principles of Kudryavtsev seem to have the best foundation. Other authors are not as consistent as he.

The biochemical tests in use are very simple; one might say, even primitive.

In order to determine whether a yeast ferments a given sugar, note is made of the gas formation in liquid medium containing the respective sugar and inoculated with the test culture.

The results of the experiment depend to a considerable extent on its setup. The result in a Dunbar tube may be positive, whereas random fermentation does not occur in an Einhorn tube. The Warburg method is yet more sensitive.

The results of the fermentation test are indicated by the sign + or —. This system contains a large source of error, since the result refers not to the organism studied, but to the experiment set up with this organism, and the experiments are an aggregate of conditions determined by the author.

For this reason sooner or later quantitative determinations should be introduced in taxonomic procedures for yeasts, as was proposed by Barnett (1957) and demonstrated by Barnett, Ingram and Swain (1956).

The same thing is true regarding the assimilation of various sources of carbon, nitrogen, and sulfur. It is established whether a given organism grows or does not grow on medium containing a certain compound as sole source of C or N in order to demonstrate whether the given compound is assimilated or not.

Moreover, the multiplication of an organism in the presence of certain compounds as sole source of C or N tells nothing about the pathway by which this compound is utilized, since there are many possibilities. In exactly the same way the causes preventing the assimilation of some compound may be different — impermeability of membrane, insufficiency of enzyme systems capable of assimilation in various structural parts, insufficiency of vitamins, etc. Furthermore, the results in assimilation experiments are determined

In large measure by the experimental setup. Besides different technical procedures (Lodder and Kreger van Rij, 1952; Barnett and Ingram, 1955; Shifrine, Phaff, and Demain, 1954), the medium employed is above all of critical importance. Here too a quantitative evaluation would be desirable (Barnett, 1957; Barnett and Ingram, 1955).

The presence or absence of enzyme systems is established by means of biochemical tests. This is the right direction, but the biochemical tests in their present-day form are not always suitable for this. Further difficulties are encountered in the need for observing the microorganism's characteristics in the culture.

The basis of the taxonomic description of new species of higher organisms are type specimens, from which the known species have been described. These type specimens are carefully preserved in museums for they are of great scientific importance. They provide an opportunity to check previous descriptions, to correct errors that have been made, and, with the further advance of natural science, to conduct supplementary studies which will prove to be necessary sooner or later.

This same role is filled in microbiology by pure cultures, from which species have been described in the past and with which newly isolated organisms are compared. Such cultures are termed type cultures. It would be desirable if every country had a collection of type cultures of such a kind.

How to preserve cultures without modifying their specific properties is a serious problem. There is no doubt that cultures are variable. This follows both from everyday experience and from theoretical considerations, since the microorganisms are grown under conditions set up by the investigator, differing from the natural ones; they multiply and continue to evolve under these conditions. Various means of preserving museum cultures employed in the taxonomy of microorganisms (10% sucrose solution according to Hansen, lyophilization, covering culture with liquid paraffin, etc.) result in slowing up the occurence of modifications, i.e., they hinder this process as much as is possible, and give quite good results. It would be erroneous, however, to make far-reaching conclusions from this regarding the stability of the microorganisms tested. Changes often take place regardless of our efforts.

I have observed that colonies of cultures, obtained from a single spore and then growing under different conditions, differed in shape, size, and color. Investigators have succeeded in modifying cultures considerably in special experiments. Thus, Kudryavtsev (1938, 1954) and Kosikov (1954) were able to convert strains not fermenting sucrose, maltose, or galactose into strains that ferment these sugars.

The preservation of museum cultures in unmodified form is today an unsolved problem.

It is necessary to perfect methods for describing species, and to increase the number of biochemical tests. Lodder and Kreger van Rij (1952) investigated fermentation capacity, mainly the assimilation of five sugars and assimilation of nitrates. Kudryavtsev (1954) studied the assimilation of many carbon sources. Wickerham (1951), Phaff (1956), and Barnett (1957) also worked with many carbon sources. The increased number of biochemical tests did not result in an increased number of species, as Lodder and Kreger van Rij believed. It would be very desirable in the description of species to include other compounds — various nitrogen and sulfur sources, vitamins, etc. Such studies are still rarely encountered since they are troublesome to conduct.

Increasing the number of biochemical tests would promote the development of the comparative biochemistry of yeasts. The qualitative and quantitative determination of enzymes, their distribution within the cell, and detailed studies of the structural elements of cell partitions require such numerous and delicate investigations that only a large group of scientists could obtain results on the large amount of material. In any event, this should be striven for. Barnett (1957) favors decreasing to a minimum the number of tests necessary to distinguish species, which would unquestionably facilitate the determination of species.

In constructing a classification system, the properties that indicate characteristics of relationship are above all necessary. They will be examined in detail below.

Problem of Constructing a Classification System.

Present-day taxonomy endeavors to group its basic units — species — into higher groups on the basis of their historical development. To accomplish this the origin of the microorganisms being classified must be known. But to comprehend the evolution of even the higher organisms is not an easy task; in microorganisms it is attended by even greater difficulties.

There exist a small number of fossil microorganisms, but even if there were many of them we could not establish their chief biochemical characteristics in any case. Thus, our conceptions of the evolution of yeasts must be based exclusively on the study of present-day organisms, and it is not surprising that there are contradictory opinions on this subject. According to Kudryavtsev (1954) the single-celled yeast fungi are a more ancient form than those that form mycelium (Endomycetales), which occupy a position on a later, lateral branch of the genealogical tree. In the opinion of other authors, for instance Gaumann (1949), the single-celled yeasts must have developed from those that form mycelium.

While there are many contradictions in the conceptions of the general trend in species development, these are fewer in the conceptions of genera, the taxonomic units immediately above species. Genera are considered to differ from one another primarily in the form of growth cycle of the organisms. The evaluation of the degree of relationship between these forms, however, not infrequently varies among different investigators.

Delimitation of species is based chiefly on biochemical characteristics. This principle is presented most consistently by Kudryavtsev (1954), who defines species as biologically separated groups of organisms

within each genus. The species adaptations in such groups are distinctly manifested in the biochemical properties. This gives to species a definite place in nature and in industry. Actually the biochemical properties determine whether an organism can live in a given medium. It is these properties that also determine the role of microorganisms in industrial production. However, they tell little of the organism's past or its origin. These may appear more clearly in the "morphological" characteristics, which are not so dependent on environmental conditions and are not so adaptive.

Kudryavtsev emphasizes that, for a description of genus, only those general morphological characteristics are suitable which are hereditary and which are correlated with the general adjustment of the organism to the conditions of its existence. In speaking of the not-as-adaptive morphological characteristics, I had in mind primarily the shape of spores, which is extremely rarely of adaptive significance.

Investigators have different opinions regarding the question of whether Saccharomyces should be separate from Zygosaccharomyces. Kudryavtsev (1954) and Kockova-Kratochvilova (1957a) separated them, in connection with a diploid or haploid phase of the forms in vegetative reproduction. As for Lodder and Kreger van Rij (1952), they do not separate such genetic pairs. Wickerham (1951) takes an intermediate position. Within Hansenula genus, he distinguished species where the great majority is chiefly diploid, transitional, or haploid.

When parallel genera are stored it is necessary to reckon with the possibility of the appearance of transitional types. I have observed in a culture of wine yeast, maintained under liquid paraffin, that paired conjugation occurred in some of the cells prior to ascus formation, but that cells from a fresh four-day culture were converted to asci for the most part without conjugation (Zsolt, 1958).*

A large number of the asporogenous yeasts are referred by Lodder and Kreger van Rij (1952) to a separate family of the Fungi imperfecti, the Cryptococcaceae. Kudryavtsev (1954) combined them with the sporogenous yeasts into the family Saccharomycetaceae, and indeed, the inclusion of asporogenous yeasts in the F. imperfecti group, which was distinguished only temporarily because of our lack of knowledge, was not justified.

The reason for the different view on yeast phylogeny lies, in my opinion, above all in the fact that we do not at present know many yeasts. Comprehensive monographs on yeasts would afford great possibilities for solving this problem. Thus, for instance, Van der Walt (1956) described a new genus, Kluyveromyces, which is distinguished by multispore asci and stands between Dipodascus and the yeasts.

In the interests of further progress, present-day genera should be thoroughly reexamined and where necessary species should be assigned to homogenous genera.

Experiments on adaptation should be continued, since they permit conclusions to be made regarding the ability to acquire various properties.

Finally, an attempt should be made to draw up a scheme of a genealogical tree within smaller groups.

All these reflections against a background of investigation of a large amount of material that describes species comprehensively will bring us closer to our ultimate purpose – a phylogenetic classification of the yeasts.

SUMMARY

A critical discussion is presented of the basic data in present-day schemes of yeast classification and of study methods.

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PARTICIPATION OF THIOBACILLUS FERROOXIDANS IN THE OXIDATION OF SULFIDE ORES IN PYRITE BEDS OF THE MIDDLE URAL

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In most of the hydrothermal deposits such elements as copper, lead, zinc, cobalt, nickel, and others, are found in the form of sulfides. Because of this, the study of oxidation and erosion of sulfide ores is of great interest.

Until quite recently, the oxidation of sulfide ores was thought to be a purely chemical process (Smirnov, 1955). Microorganism participation was elucidated mainly by American investigators who, in 1947, isolated a new species of autotrophic organism, Thiobacillus ferrooxidans, capable of oxidizing oxide compounds such as iron and sulfur (Colmer, Hinkle, 1947). The widespread distribution of this organism in nature indicates the great role it plays in the oxidation of sulfide ores. Bryner, Beck, Davis, Wilson (1954), and Bryner and Jamerson (1958) isolated T. ferrooxidans from the waters of copper mines of the Bingham Canyon (USA) and Cananea (Mexico) and demonstrated in laboratory tests that under the action of this organism oxidation of sulfide ores is several times faster.

We isolated this organism from the Akhtal' polymetallic deposit in the Armenian SSR (Ivanov, Lyalikova, Kuznetsov, 1958).

The object of the present work was to study the propagation and conditions of development of T. ferrooxidans in a number of sulfide deposits in Middle Ural and to elucidate the role which this organism plays in the oxidation of sulfide ores in these deposits.

Deposits of cuprous and polymetallic pyrite ores are in the main related to metamorphosed rocks of the greenstone stratum which runs along the whole of the Ural east of the divide. We investigated four deposits: Degtyar, Krasnogvardeiskii, Third International and Upper Pyshma.

The Degtyar copper-pyrite deposit is the most typical and largest of the pyrite deposits of Middle Ural (Varkhomeev, 1935; Ivanov, 1945). It is lens-shaped and occurs at the point of contact with the quartz-albite porphyroids and quartz-sericite slates. The Degtyar deposit is distinguished by its length, 4800 m along the course, and by the severe wrinkling of the enclosing slates and of the vein itself. The ore is copper and sulfur pyrite but the sides of the bed are richer in copper. As a result of erosion, the deposit has acquired a zonal structure. Below the oxidized zone is a zone of the secondary enrichment of ores; at a depth of 100 m the content of secondary sulfides decreases and at a depth of 170–190 m only primary ores are present.

We began our microbiological investigation of the Degtyar deposit with an examination of the dumping grounds of old ores, as we were interested in the question as to whether the microorganisms participated in the disintegration to which ores which had lain for several years on the diurnal surface are subjected. Specimens taken from dumping grounds or neighboring sections had a high active acidity; the pH of the investigated specimens ranged from 1.2–4.8. This acidity resulted in the complete burning out and destruction of the surrounding vegetation. The development of T. ferrooxidans was observed upon sowing most of the old, completely destroyed ore specimens as well as on sowing ores of comparatively recent extraction which had lain on the surface for nearly two years. The number of T. ferrooxidans ranged from 1000 to 10,000 cells per one gram of rock. The results obtained indicate that this organism participates in the oxidation in dumping grounds.

A complete investigation was carried out on specimens from the 250-meter level of pit No. 2, the results of which are presented in Table 1.

Table 1. Distribution of Thiobacillus ferrooxidans in the Degtyar Deposit

Places from which specimens were taken	pH	Number of T. ferrooxidans cells in 1 ml of water or 1 g of rock
Water from cracks in the quartz-albite porphyroids	3.71	100
Crack behind the second drop in the southern field drift	2.86	0
Clogged up horizontal crack behind the second drop	2.57	100
Drippings from the drift wall at the point of contact of ores with rocks in the southern drop	3.14	100
Water from the trench in the third drop	1.72	10,000
Water from the crack in the ore field	—	1,000
Freshly extracted ore from the 108th chamber of the fifth drop	—	0
Water from the second southern rising drift	1.5	1,000
Water from the northern field drift behind the first northern drop	2.43	1,000
Water from the northern field drift behind the second northern drop	—	10,000
Water from cracks in the greenstone stratum of the enclosing rock	3.4	0
Freshly extracted ore	—	0
Second northern drop, contact of rock with the ore body	—	100
Ore from the 130th inflammable level	—	0

Of 14 investigated specimens, T. ferrooxidans was present in nine. It was found in water of the crack

running along the surrounding rocks and in destroyed slates at the point of contact with the ore, but the largest number was in the water flowing through the cracks in the ore body as well as in the water that ran down the drift. The organism was not found in specimens of freshly extracted ore.

The Degtyar deposit has a considerable water supply; moreover, the ore waters contain a large amount of copper sulfate. For instance, the waters of pit No. 2, which we investigated, contained an average of 1.27 g/l of copper and 1.35 g/l of sulfuric acid. When chalcopyrite is oxidized the copper passes into solution by the following reaction:

$$CuFeS_2 + 4O_2 = CuSO_4 + FeSO_4.$$

This reaction can take place by purely chemical means but it is considerably accelerated by the action of T. ferrooxidans. The action of this organism is not confined to the straightforward oxidation of sulfides. It had long been known that the oxidation of chalcopyrite as well as of other sulfides is greatly accelerated by ferric sulfate:

$$CuFeS_2 + 2Fe_2(SO_4)_3 + 2H_2O + 3O_2 = CuSO_4 + 5FeSO_4 + 2H_2SO_4.$$

As a result of the interaction with the sulfide, the ferric oxide is converted into a ferrous form, which, according to Academician Smirnov, is capable of being oxidized only in the absence of sulfuric acid. In actual fact, ferrous sulfate, although resistant to chemical oxidation in an acid medium, is easily converted into an oxide form by the action of T. ferrooxidans. Thus, this organism may be the indirect means of accelerating the leaching of copper by forming a powerful oxidizing agent, $Fe_2(SO_4)_3$.

In the Degtyar mine, in order to extract the copper which had passed into solution a special cemented assembly was constructed on which the copper is deposited by means of the conversion reaction with iron. This reaction takes place in a special block of trenches into which the water pumped out of the pit and passed through sedimentation tanks flows.

The oxidizing-reducing conditions, the acidity and number of T. ferrooxidans were determined at different stages of extraction of copper hydroxide. The results are embodied in Table 2.

The table clearly shows that the magnitude of the oxidizing-reducing potential in the water of the assembly depends on the concentrations of ferrous and ferric oxide. The water which flows in from the pit contains a considerable amount of ferrous oxide and consequently has a low oxidizing-reducing potential. In the sedimentation tank the ferrous oxide of the mine waters is converted by T. ferrooxidans into ferric oxide and this results in a higher potential. When parings of sheet iron are added to the water in the troughs the following reaction takes place:

$$CuSO_4 + Fe = FeSO_4 + Cu.$$

The ferrous oxide which is formed again reduces the potential. As in the troughs there is a high content of ferrous oxide and optimum acidity for T. ferrooxidans, exceptionally favorable conditions are produced there for its development. An analysis has demonstrated that the number of T. ferrooxidans in 1 g of copper hydroxide concentrate exceeds one million cells.

Table 2. Conversion of the Oxidizing-Reducing Potential and Acidity of the Medium in the Cementation of Copper

Place from which the water specimen was taken	pH	Eh	rH	Number of T. ferrooxidans in 1 ml of water
From the pit before passing through the sedimentation tank	2.93	340	17.58	10,000
From the sedimentation tank	2.74	—	—	1,000
After passing through the sedimentation and before the addition of iron	2.62	605	26.1	—
First trough with iron	4.1	250	16.82	—
The terminal end of the troughs with iron	2	250	12.62	10,000

In the Krasnogvardeisk deposit, which we also investigated, a similar assembly exists for the deposition of copper from mine water. The Krasnogvardeisk pyrite deposit is situated in Krasnoural, 148 km northeast of Sverdlovsk. The deposit was thoroughly investigated by Amiraslanov in 1934. It is located at the junction of greenstone rock and quartz-sericitic and chloritic slates. Morphologically the Krasnogvardeisk deposit consists of several lens-shaped ore bodies of which the main lens is the thickest. The mineralogical composition is as follows: pyrite 65–80%, chalcopyrite 8–10%, sphalerite 1–2%. (Bornite and tennantite are rarely encountered.)

The development of oxidizing processes in the highly sulfurous ore of the Krasnogvardeisk deposit in many cases resulted in spontaneous combustion (Enikeev and Sindarovskii, 1947).

Because of this, in order to elucidate the role of T. ferrooxidans in the self-heating of ores we investigated the propagation of this organism in the inflammable sections. The results are presented in Table 3. As will be observed from the table, T. ferrooxidans was found in all specimens except in ores from old dumping grounds. The organisms were found in large numbers in waters which flowed through the inflammable regions that had been silted up with clay pulp to extinguish a fire or simply as a preventive measure. An active strain of T. ferrooxidans was isolated from ores in which the process of self-heating had begun.

This conclusively demonstrates that T. ferrooxidans participates in the initial stages of the self-heating of ores. The further heating which may result in fire is apparently due to the chemical reactions of oxidation of sulfur compounds.

The third pyrite deposit we investigated was the Third International situated 8 km north of the town Nizhnii Tagil and near the San Donato station. It lies in the Tagil stratum of greenstone rock, the ore bodies being located in quartz-sericite slates. The deposit consists of three ore zones: the eastern, called the Fifteenth Anniversary of October, the middle—Shmidt zone and the western—Ceonyi deposit. The first two

able 3. Propagation of Thiobacillus ferrooxidans in Krasnogvardeisk Deposit

Place from which specimen was taken	pH	Number of T. ferrooxidans in 1 ml of water or 1 g of ore
evel 304, 5 blocks, water from section silted up to extinguish fire	1.55	100–1000
evel 304, third southern lens, water and rusty incrustations on the ore	3.4	+
evel 244, main lens, 6 blocks, water from section subjected to fire prevention treatment	1.7	10,000
evel 244, main lens, 7 blocks, water from the section under preventive treatment	1.6	100,000
evel 237, main lens, 3 blocks, heated ore.	—	+
emented assembly, middle of the trough with iron	2.85	10,000
ried concentrate of copper hydroxide	—	+
res from old dumping grounds	—	0

Note: + signifies that T. ferrooxidans is present.

ones belong to the copper-zinc deposits and the Ceonyi o the sulfur-pyrite deposits. The ores are divided nto impregnated, which have the appearance of quartz-ericite slates impregnated with sulfides, and the con-inuous which consists of pyrite, sphalerite, chalco-yrite and bornite.

As already demonstrated by Mazurin (1955) the verage composition of continuous ores is as follows: yrite–30%, sphalerite–23%, chalcopyrite–12%.

This deposit was investigated at a level of 355 m; moreover, particular attention was paid to the pres-nce of T. ferrooxidans in ore specimens. The re-sults are presented in Table 4.

Thus, in the course of our investigation of the Third nternational deposit, T. ferrooxidans was isolated on sowing scrapings from specimens of fresh, slightly oxidized ores. Although T. ferrooxidans plays a defi-nite role in the oxidation of certain sections of the leposit, its importance in the oxidation of ores of this leposit is probably insignificant. There is no cemented assembly at the mine as the waters contain only a small amount of copper. This is probably due to the act that the massive and impregnated ores which nake up the bed have a great resistance to chemical nd bacterial oxidation.

Table 4. Spread of Thiobacillus ferrooxidans in the Third International Deposit

Place from which specimen was taken	pH	Presence of T. ferro-oxidans
Zone 15th Ann. of October, lens 7-B, massive slightly cuprous pyrite	—	+
Zone 15th Ann. of October, 17th layer, lens 7-B, massive zinc-copper pyrite	—	+
Zone 15th Ann. of October, lens 7-B, massive zinc-copper pyrite	—	+
Zone 15th Ann. of October, lens 7-A, massive zinc-copper pyrite	—	0
Zone 15th Ann. of October, lens 12, water	3.5	1000
Zone 15th Ann. of October, lens 12, impregnated zinc-copper ore	—	0
Ore zone Shmidt, water from lens 10	0.6	1000
Zone 15th Ann. of October, water from lens 3A	4.9	0
Zone 15th Ann. of October, water from the trench	3.2	+
Zone 15th Ann. of October, lens 3, impregnated zinc-copper ore	—	+

Note: + signifies that T. ferrooxidans is present.

The Pyshma deposit is situated 15 km from Sverd-lovsk. The deposit consists of 23 ore zones of which 19 are industrial. The ore zones comprise saturated sulfides and chlorite slates which lie among the por-phyrites. The ore consists of pyrite, chalcopyrite, and molybdenite; the western section also contains cobalt. The upper part of the deposit is oxidized. The depth of oxidation ranges from 40 to 65–70 m (Mala-khov, 1941).

We investigated this deposit at levels of 55 and 85 m. It differs from those already described in that the pit waters give a neutral reaction and there is an almost total absence of T. ferrooxidans. On examining selec-ted specimens the organism was found only in a speci-men of copper salt incrustations on the drift wall. The organisms were also isolated from one specimen of oxidized ore from an old dump. Although the reasons for the almost total absence of T. ferrooxidans are not quite clear it may be assumed that it is probably due to the fact that the ore body has very few cracks. It should here be remarked that the ore body of the Degtyar deposit where T. ferrooxidans is present in large numbers is highly fissured. Moreover, the fact that the Pyshma deposit was investigated at levels of 55 and 85 m which are within the limits or border of the oxidation zone may be of significance as all the other deposits were investigated at zones of primary ores.

SUMMARY

1. Thiobacillus ferrooxidans has been isolated from all the four investigated pyrite deposits of Middle Ural. It plays a great role in the oxidation of ores in the Degtyar and Krasnoural deposits where it numbers 10,000 cells in 1 ml of mine water.

2. The universal occurrence of T. ferrooxidans in the waters of inflammable districts of the Krasnoural deposit suggests that it may participate in the initial stages of the self-heating of ores.

3. The lesser occurrence of T. ferrooxidans in the Third International deposit and its almost total absence in Pyshma is probably associated with the geology of these deposits. Of particular importance is the cracked structure of the ore body.

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ON ACTINOPHAGE ADSORPTION BY SUSCEPTIBLE ACTINOMYCETES

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The process of phage multiplication is known to consist of several stages: adsorption of phage on the cell, penetration of DNA of the phage particle into the microbial cell, then the onset of the complex process of vegetative phage growth ending in formation of the mature particles, and finally the rupture of the cell with liberation of phage particles.

The adsorption of bacteriophages by the bacterial cell has been studied in the most detail and there is an extensive literature on this subject.

The adsorption of actinophages by susceptible actinomycetes, however, has been studied very little. This question is touched upon in the work of Mach (1958), who made an electron microscope study of actinophage adsorption by germinating spores and mycelium of actinomycetes (see also work of Gilmour, Noller and Watkins, 1959).

The purpose of the present paper was to study the adsorption capacity of two species of actinomycetes, Actinomycetes olivaceus and Actinomycetes streptomycini Kras., with respect to actinophages active against them. The extent of adsorption in relation to age of the host culture was studied, and also the effect of ultrasound on the adsorption capacity of the mycelium.

The inclusion in our studies of questions regarding the effect of ultrasonic waves on adsorption capacity of mycelium is due to the fact that ultrasound causes substantial changes under certain conditions in the physical and colloidal state of cell membranes and films, and also in the activity of enzymes localized in surface layers of animal and plant cells. Moreover, an increased sensitivity to disinfectants and antibiotics is noted in sound-treated microbes (El'piner, 1955).

METHODS

The following cultures and actinophages were employed in the experiments: A. streptomycini Kras. B and B/I and actinophages active against them of Types I and II described by Rautenshtein (1954), A. olivaceus No. 1255 and actinophage 8238 active against it, which was isolated and described by Khavina and Rautenshtein (1958).

Cultivation of experimental cultures and actinophage multiplication in them was carried out in fish broth (2% fish extract and 2% glucose in tap water) on a shaker (120 rpm) at 26–27°.

Adsorption capacity of actinomycetes was determined in the following way. Actinophage was added to 2 ml of spore suspension of definite concentration in fish broth or of mycelial mass in the same medium. Phage was added on the basis of 1–4 particles per spore or its sprout. Spores were previously filtered through a specially set up pipette with a cotton plug at the end, and then counted in a Goryaev chamber.

Duration of adsorption in all experiments was 1 hour. Adsorption took place at 5° and 26° with periodic mixing. The mycelium, or spores, were precipitated by centrifuging for 10 minutes at 3000 rpm. The number of adsorbed phage was determined by the difference between the titer at the moment of adding phage to the culture and the titer in the supernatant liquid.

Titration was carried out by the two-layer method. The optimal number for titration and age of test-culture mycelium, added to the upper layer, were determined empirically for each experimental culture.

To determine phage titer in A. olivaceus a 24-hour culture grown in fish broth on a shaker was employed, to determine phage titer in A. streptomycini a 48-hour culture in the same medium was employed.

As is known, it is recommended in determining adsorption of bacteriophage (Adams, 1950) to dilute 100 times the phage-bacteria system after a definite period of contact in order to stop adsorption.

Table 1. Effect on Adsorption of Diluting Actinophage–Actinomycete System (Actinomyces streptomycini B/I and Type II Actinophage)

Culture age in hrs	No. (%) of actinophage adsorbed without dilution	No. (%) of actinophage adsorbed with 1:100 dilution	
		meat-peptone broth	distilled water
18	80	90	
24	98	98	98
36	98	97.5	96

The studies we conducted, whose results are shown in Table 1, showed that under our experimental conditions the adsorption of actinophage was virtually the same in the diluted and undiluted actinophage-actinomycete system.

EXPERIMENTAL

Adsorption capacity was determined in spores, in their 6–9 hour sprouts, in 12–24 hour mycelium in a state of formation, and in 48–96 hour mycelium in a state of decomposition and autolysis.

The comparative microscopic observation of spore germination and mycelium growth in the experimental cultures, A. streptomycini B/I and A. olivaceus 1255, showed the following: Spore germination does not proceed at one pace. At 4 hours after seeding in fish broth a swelling of the spores is observed and only a small number begin to germinate. At 6 hours an absolute majority of the spores germinates and a small sprout beginning to branch is formed (Figure 1). At 8 hours stronger branching is observed, and at 12 hours small microcolonies are being formed (Figure 2).

No material differences in the nature of the growth of A. streptomycini B/I and A. olivaceus 1255 are noted during this period of time. A difference in the nature of the growth of these cultures sets in distinctly in the 20-hour and older cultures.

A. streptomycini B/I forms in fish broth branching, noncompact microcolonies, whereas in A. olivaceus No. 1255 the central part of colonies is more compact (Figures 3, 4).

Experimental results on adsorption capacity are presented in Tables 2 and 3.

Data on adsorption of actinophage No. 8238 by A. olivaceus No. 1255 are given in Table 2.

It is seen from Table 2 that spores and their sprouts 4–6 hours in age are able to adsorb actinophage specific for the given culture. The extent of this adsorption, however, insofar as our technique enables us to determine, is not so great as in older mycelium (12–24 hours). Maximal adsorption in all our experiments was observed in 20–24 hour mycelium. In 48-hour and older mycelium the adsorption capacity drops abruptly.

It is also evident from Table 2 that the extent of adsorption is generally higher at 26° than at 5°.

Special experiments to determine the effect of temperature on adsorption showed that up to 37° it proceeds as it does at 26°. With further rise in temperature, however, decrease in the extent of adsorption is observed. Since preliminary experiments showed that adsorption proceeds more actively at 26°, all subsequent experiments on adsorption were conducted at this temperature.

In Table 3 are given the experimental results on adsorption by A. streptomycini B/I of Type II actinophage specific for this culture.

As Table 3 shows, the same features of adsorption observed in A. olivaceus and phage No. 8238 are noted also in this actinophage+actinomycete complex. In this case too the maximal absorption capacity was in 20–24 hour mycelium; it was reduced in 48- and 72-hour mycelium.

Demonstration of the fact that the extent of adsorption depends on the physiological age of mycelium, and not on its total biomass that usually increases with age during the first three days of growth, is indicated by data from special experiments shown in Table 4.

As seen from Table 4, maximal adsorption was observed in cultures 20–24 hours of age whose biomass was less than in 42–72 hour cultures.

In the next experiments the problem was to determine the effect of the host culture on adsorption capacity. For this purpose we employed A. streptomycini Type I actinophage and cultures of A. streptomycini B and A. olivaceus 1255.

We selected Type I actinophage since, as was shown previously, it lysed not only A. streptomycini B but also A. olivaceus 1255, whereas Type II actinophage did not lyse A. olivaceus 1255, and actinophage No. 8238 did not lyse A. streptomycini (Khavina and Rautenshtein, 1958). The Type I actinophage employed in the experiments had multiplied on A. streptomycini B for a number of years. The titer of this phage, prepared by us for the experiments, was on the average $4 \cdot 10^8$ in the test-culture A. streptomycini B. On checking the titer of this phage by the two-layer method on a culture of A. olivaceus 1255, however, it was found that not only was it considerably lower, but that

Table 2. Adsorption of Actinophage 8238 by Actinomyces olivaceus No. 1255

Expt. no.	Culture age (hrs)	Phage titer before adsorption	Phage titer after 1-hr contact with culture at 26°	Phage titer after 1-hr contact with culture at 5°	No. of adsorbed phage particles (%) at 26°	No. of adsorbed phage particles (%) at 5°
1	spores	$4 \cdot 10^8$	$3 \cdot 10^8$	not detd.	25	—
	6	$2 \cdot 10^8$	$1.2 \cdot 10^8$	$1.6 \cdot 10^8$	40	20
	9	$2 \cdot 10^8$	$1.6 \cdot 10^8$	$1.2 \cdot 10^8$	20	40
	12	$2 \cdot 10^8$	$2 \cdot 10^7$	not detd.	90	—
2	spores	$6 \cdot 10^8$	$1,2 \cdot 10^8$	not detd.	80	—
	4	$2 \cdot 10^8$	$1,2 \cdot 10^8$	$1.2 \cdot 10^8$	40	40
	6	$2 \cdot 10^8$	$1,2 \cdot 10^8$	not detd.	40	—
	12	$2 \cdot 10^8$	$1,2 \cdot 10^7$	$4 \cdot 10^7$	94	80
	20	$2 \cdot 10^8$	$4 \cdot 10^4$	$1.6 \cdot 10^5$	99.9	99.2
3	20	$4 \cdot 10^6$	$4 \cdot 10^3$	$8 \cdot 10^4$	99.9	98
	24	$4 \cdot 10^6$	$4 \cdot 10^3$	$4 \cdot 10^5$	99.9	90
	48	$6 \cdot 10^6$	$1,2 \cdot 10^6$	$1.2 \cdot 10^6$	80	80
4	20	$1 \cdot 10^7$	$1,2 \cdot 10^4$	not detd.	99.8	—
	24	$1 \cdot 10^7$	$1,2 \cdot 10^4$	$2 \cdot 10^5$	99.8	98
	48	$1 \cdot 10^7$	$8 \cdot 10^6$	not detd.	20	—
	96	$6 \cdot 10^7$	$4 \cdot 10^7$	not detd.	33.4	—

Table 3. Adsorption of Type II Actinomyces streptomycini Actinophage by Actinomyces streptomycini B/I

Expt. no.	Culture age (hrs)	Phage titer before adsorption	Phage titer after 1-hr contact with culture	No. of adsorbed phage particles (%)
1	4	$2 \cdot 10^7$	$3.2 \cdot 10^7$	0
	9	$2 \cdot 10^7$	$8 \cdot 10^6$	60.0
	12	$2 \cdot 10^7$	$3.2 \cdot 10^5$	98.4
	24	$2 \cdot 10^7$	$2.4 \cdot 10^5$	98.8
	48	$1 \cdot 10^8$	$4 \cdot 10^6$	96.0
2	6	$2 \cdot 10^8$	$1.2 \cdot 10^8$	40.0
	9	$2 \cdot 10^8$	$1.2 \cdot 10^8$	40.0
	12	$2 \cdot 10^8$	$4 \cdot 10^6$	98.0
	20	$2 \cdot 10^8$	$4 \cdot 10^5$	99.8
	24	$2 \cdot 10^8$	$2 \cdot 10^5$	99.9
	48	$2 \cdot 10^8$	$8 \cdot 10^6$	96.0
	72	$1.3 \cdot 10^{10}$	$2.8 \cdot 10^9$	79.5
3	24	$4 \cdot 10^9$	$8 \cdot 10^7$	98.0
	42	$1 \cdot 10^9$	$6 \cdot 10^8$	40.0
	70	$1 \cdot 10^9$	$8 \cdot 10^8$	20.0

Table 4. Extent of Actinophage Adsorption by Mycelium in Relation to Age and Total Mass of the Mycelium

Culture on which phage was adsorbed	Phage adsorbed	Culture age (hrs)	Phage titer before adsorption	Phage titer after 1-hr contact with mycelium	No. of adsorbed phage particles (%)	Mycelium dry wt. in g / 100 ml cult. fl.	No. of adsorbed phage particles per g of dry mycelium
Act. streptomycini B	A. streptomycini Type II	18	$4 \cdot 10^9$	$8 \cdot 10^8$	80	0.16	$2 \cdot 10^{12}$
" "	" "	24	$4 \cdot 10^9$	$8 \cdot 10^7$	98	0.21	$1.8 \cdot 10^{12}$
" "	" "	42	$1 \cdot 10^9$	$6 \cdot 10^8$	40	0.49	$8 \cdot 10^{10}$
" "	" "	72	$1 \cdot 10^9$	$8 \cdot 10^8$	20	0.25	$8 \cdot 10^{10}$
Act. olivaceus 1255	A. olivaceus 8238	20	$1 \cdot 10^7$	$1.2 \cdot 10^4$	99.8	0.25	$3.9 \cdot 10^9$
" " "	" " "	24	$1 \cdot 10^7$	$1.2 \cdot 10^4$	99.8	0.25	$3.9 \cdot 10^9$
" " "	" " "	48	$1 \cdot 10^7$	$8 \cdot 10^6$	20	0.29	$6.8 \cdot 10^8$

Table 5. Adsorption of Type I Actinophage on Mycelium of Actinomyces streptomycini B and Actinomyces olivaceus 1255 Cultures

Expt. No.	Culture on which actinophage was prepared	Culture on which actinophage was adsorbed	Culture on which actinophage was titrated	Phage titer before absorption	Phage titer after 1-hr contact with culture	No. of adsorbed phage particles (%)
1	Act. streptomycini B	Act. streptomycini B	Act. streptomycini B	$4 \cdot 10^8$	$8 \cdot 10^6$	98.0
2	The same	The same	The same	$4 \cdot 10^7$	$2,4 \cdot 10^5$	99
3	" "	Act. olivaceus 1255	" "	$4 \cdot 10^8$	$8 \cdot 10^7$	80.0
4	" "	" "	" "	$4 \cdot 10^8$	$8 \cdot 10^7$	80.0
5	" "	" "	" "	$4 \cdot 10^8$	$3.2 \cdot 10^8$	20.0
6	" "	" "	" "	$4 \cdot 10^7$	$3.2 \cdot 10^7$	20.0
7	Act. olivaceus 1255	Act. streptomycini B	Act. olivaceus 1255	$2 \cdot 10^8$	$1.4 \cdot 10^7$	93.0
8	" "	Act. olivaceus 1255	" "	$4 \cdot 10^8$	$2 \cdot 10^8$	50.0
9	" "	" "	" "	$4 \cdot 10^8$	$3 \cdot 10^8$	25.0
10	" "	" "	" "	$4 \cdot 10^8$	$1.6 \cdot 10^8$	60.0
11	" "	" "	" "	$4 \cdot 10^8$	$2.4 \cdot 10^8$	40.0
12	" "	" "	" "	$4 \cdot 10^8$	$1.6 \cdot 10^8$	60.0

Table 6. Adsorption by Sound-treated Mycelium of Actinophages Actinomyces olivaceus 8238 and Actinomyces streptomycini Type II

Expt. No.	Culture	Duration of treatment	Phage titer before adsorption	Phage titer after 1-hr contact with culture
1	Act. olivaceus 1255	Not treated	$1.2 \cdot 10^8$	$1.2 \cdot 10^5$
2	" "	" "	$6 \cdot 10^8$	$1.6 \cdot 10^5$
3	" "	" "	$4 \cdot 10^8$	$4 \cdot 10^6$
4	" "	5 sec	$1.4 \cdot 10^8$	$4 \cdot 10^4$
5	" "	5 sec	$6 \cdot 10^8$	$8 \cdot 10^4$
6	" "	10 sec	$1.4 \cdot 10^8$	$8 \cdot 10^4$
7	" "	10 sec	$4 \cdot 10^8$	$8 \cdot 10^5$
8	" "	30 sec	$1.4 \cdot 10^8$	$8 \cdot 10^4$
9	" "	3 min	$1.4 \cdot 10^8$	$8 \cdot 10^4$
10	" "	3 min	$4 \cdot 10^8$	$8 \cdot 10^5$
11	" "	10 min	$4 \cdot 10^8$	$8 \cdot 10^5$
12	" "	10 min	$4 \cdot 10^8$	$2.4 \cdot 10^5$
13	" "	20 min	$4 \cdot 10^8$	$2.4 \cdot 10^5$
14	Act. streptomycini B	Not treated	$2 \cdot 10^8$	$4 \cdot 10^5$
15	" "	5 sec	$2 \cdot 10^8$	$4 \cdot 10^5$
16	" "	10 sec	$2 \cdot 10^8$	$4 \cdot 10^5$
17	" "	3 min	$2 \cdot 10^8$	$4 \cdot 10^5$
18	" "	10 min	$2 \cdot 10^8$	$8 \cdot 10^5$

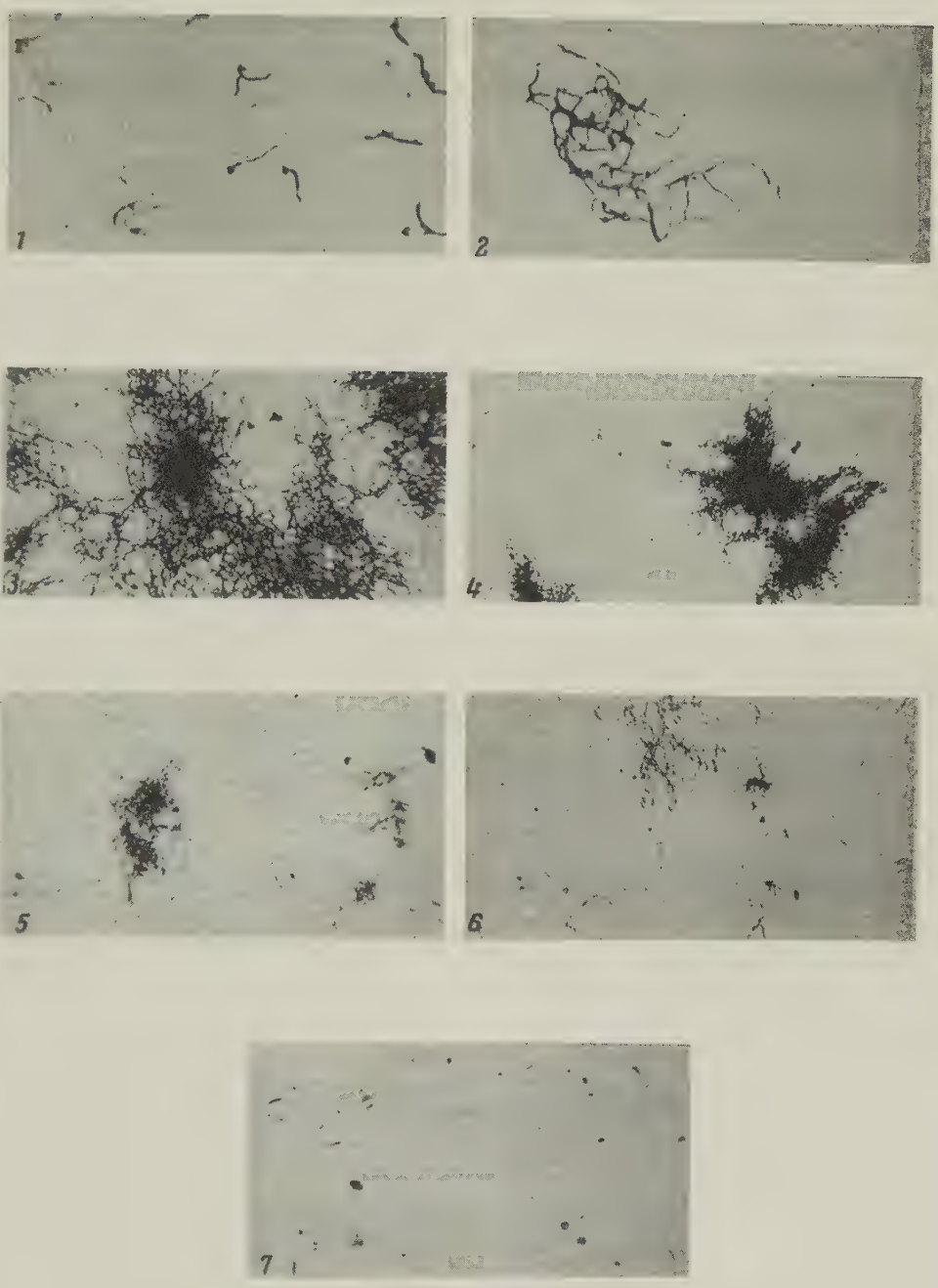

Fig. 1. 6-hour Actinomyces streptomycini B/I; mag. 350x.
Fig. 2. 12-hour Actinomyces streptomycini B/I; mag. 350x.
Fig. 3. 20-hour Actinomyces streptomycini B/I; mag. 120x.
Fig. 4. 20-hour Actinomyces olivaceus 1255; mag. 120x.
Fig. 5. 20-hour Actinomyces olivaceus 1255 after sound-treatment for 5 sec.; mag. 120x.
Fig. 6. 20-hour Actinomyces streptomycini B/I after sound-treatment for 5 sec.; mag. 120x.
Fig. 7. 20-hour Actinomyces olivaceus after sound-treatment for 3 minutes; mag. 350x.

negative colonies were scarcely apparent in a number of cases. Therefore, in order to raise the activity of Type I phage toward A. olivaceus 1255, its multiplication was carried out on this culture. Even after the second passage the titer of Type I actinophage in the A. olivaceus culture increased considerably and negative colonies assumed their characteristic dimensions. After the fourth passage in the A. olivaceus culture a phage lysate was obtained with titer $5 \cdot 10^9$ on the A. olivaceus 1255 culture and $4 \cdot 10^8$ on the A. streptomycini B culture.

It is of importance to note that this actinophage, after 2-month storage under refrigeration, had a sharply reduced lytic activity toward A. streptomycini B, but fully retained its activity toward A. olivaceus 1255 on which it had multiplied. Thus, its titer in A. olivaceus 1255 was $2 \cdot 10^9$ and in A. streptomycini B 10^4–10^5, the size of negative colonies was much smaller than usual in the latter culture with the colony outlines indistinct. These data show that the culture on which it multiplies often exerts a substantial effect on the actinophage lytic properties, which has been shown in experiments with other actinophages (Rautenshtein, 1959; Alikhanyan and Il'ina, 1959).

Freshly prepared phage lysates of Type I actinophage that had multiplied both on A. streptomycini B and on A. olivaceus 1255 were employed in the experiments on adsorption.

In Table 5 are given the results of experiments on adsorption of A. streptomycini Type I phage by 20-hour mycelium from cultures of A. streptomycini B and A. olivaceus 1255.

It is seen from Table 5 that A. streptomycini Type I actinophage was adsorbed better by the A. streptomycini B culture than by the A. olivaceus 1255 culture susceptible to it. Even in the case where this actinophage multiplied in the A. olivaceus culture, the extent of adsorption by the A. streptomycini culture was greater.

By comparing Table 2 with Table 5 it is seen that 20-hour mycelium of A. olivaceus 1255 does not adsorb the actinophage No. 8238 specific for it and the Type I actinophage to an equal extent.

Adsorption of Actinophages by Mycelium Treated with Ultrasound

Cultures of A. streptomycini B/I and Type II actinophage active against it, and A. olivaceus No. 1255 and actinophage No. 8238 specific for it, were used in the experiments.

Mycelium, 20 hours old, grown in fish broth on a shaker was treated with ultrasound. Two milliliters of such mycelium was put in glass tubes and treated with sound in a piezoquartz generator (vibration frequency 750 kc, ultrasound intensity ~15 w/cm^2) with continuous cooling. Duration of the treatment varied from 5 seconds to 20 minutes. Actinophage was added to the mycelium immediately after sound-treatment. Determination of unabsorbed phage was determined just as in experiments without sound-treatment.

The appearance of the mycelium after sound-treatment for 5 seconds is shown in Figures 5 and 6, and after treatment for 3 minutes in Fig. 7.

As seen from these figures, even 5-second sound-treatment produces great disintegration of mycelium into small fragments. A. olivaceus retains the compact central part of the microcolony in this case. After 3-minute sound-treatment these compact formations are lacking; only very fine mycelial fragments are seen in the preparations. After 10–20-minute treatment the mycelium is almost completely disintegrated. However, seedings made from this material onto nutrient medium show that even it contains viable fragments. The data from experiments on adsorption capacity of mycelial fragments obtained by the action of ultrasound are presented in Table 6.

It is seen from Table 6 that mycelial fragments, obtained after sound-treatment for 5 seconds to 20 minutes, do not lose the capacity to adsorb phage. In some experiments A. olivaceus mycelium treated for 5 seconds adsorbed considerably more actinophage than the control untreated mycelium. This is evidently related to the fact that not only does a certain increase in surface of the actinomycetes treated with ultrasound occur, but it is possible that their adsorption capacity increases.

SUMMARY

1. Actinomyces olivaceus spores and their sprouts 4–6 hours old can adsorb specific actinophage. The adsorption is much less, however, than in older mycelium. Maximum adsorption was found in 20–24-hour mycelium in A. olivaceus and Actinomyces streptomycini cultures. In 48-hour and older mycelium the adsorption capacity drops sharply compared to 20–24-hour mycelium.

2. At 26 deg adsorption is generally greater than at 5°. At a temperature over 37° adsorption is reduced.

3. The extent of adsorption of actinophage depends on the host culture. Thus, A. streptomycini B adsorbs Type I actinophage more actively than A. olivaceus 1255, susceptible to this phage. A. olivaceus 1255 does not adsorb A. olivaceus 8238 actinophage and A. streptomycini Type I actinophage to the same extent.

4. Mycelial fragments, formed by the action of ultrasound, can adsorb actinophage. In some experiments the sound-treated mycelium adsorbed considerably more actinophage than the untreated control.

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EFFECT OF FIXATION ON MORPHOLOGY OF BACILLUS MYCOIDES BACTERIOPHAGE

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During study of the structure of particles of Bacillus mycoides phage we encountered the phenomenon of great morphological diversity even in the same preparation. Some particles had a dense head and a tail uniform in width throughout its length; others, despite the fact that the head remained dense to electrons, had a tail whose distal end (about 1/4) was much finer than the rest of it (denuding of tail core) (Fig. 1); and finally there were particles whose heads appeared depressed, diffuse, devoid of inner contents and hence more transparent to electrons (head membranes) (Fig. 2).

It should be noted that the ratio between damaged phage particles and ones where the external appearance was more or less preserved varied in different preparations. Some preparations contained essentially almost normal phage particles, in others the particles were mostly deformed and partially damaged.

These facts induced us to conduct studies with the object of determining the causes producing such a diversity of forms of the phage corpuscle. These causes are very probably multiple. On the one hand it may be assumed that the phage lysate contains a certain number of immature or damaged phage forms and, on the other, that deformation and damage occur in the process of making the preparations.

MATERIALS AND METHODS

In the study we employed B. mycoides grown on meat-peptone media at 26-27° and the phage to this culture described by us in a previous study (Kriss and Tikhonenko, 1957) and the designated phage No. 1. The broth phage lysate of this culture was filtered through a Seitz filter with asbestos pads. The filtered phage lysate was treated in the following way: it was either purified by the method of drop dialysis for 24 hours at 37° (Kriss, Biryuzova, and Zolkover, 1948) with subsequent fixation, or first fixed by a given method and then dialyzed, or fixation and purification by the dialysis method were conducted simultaneously.

The following fixing agents were used: half-saturated picric acid solution (1 volume saturated picric acid to 1 volume phage lysate), 1% trichloroacetic acid solution, vapors and 1% solution of OsO_4 in veronal-acetate buffer at pH 7.2 for different lengths of time—from 10 minutes to 24 hours (fixation according to Palade, 1952), vapors and 2-4% neutral formalin solution for different lengths of time—from 10 minutes to 5 days. All the fixing agents enumerated were employed in the case of fixation prior to dialysis. The agents used in the form of a solution were added directly to the phage lysate up to the required concentration. For fixation by vapors of OsO_4 and formalin, the unpurified phage lysate was first fixed under a bell jar and then purified by the method of drop dialysis. Phage fixation after dialysis was conducted only with OsO_4 and formalin in vapor form. Fixation during dialysis was conducted with formalin vapors. For this purpose a disk of filter paper, moistened with formalin, was placed on the inner surface of the lid of the Petri dish in which dialysis was occurring.

Fixed and unfixed preparations of phage No. 1, purified by the drop dialysis method in all cases, were dried in air and dusted with chrome. Preparations were examined in electron microscope UEM-100 at 60 kv.

RESULTS AND DISCUSSION

Titration of phage lysate before and after dialysis showed that phage No. 1 does not lose lytic activity in the process of dialysis (Table 1). This fact compels us to assume that the gradual change in osmotic pressure during dialysis does not damage the phage particles. As will be shown below, this assumption is confirmed by the morphological pictures of phage particles obtained after fixation.

Thus there remains one more step in the process of making preparations, which is associated with dehydration and the action of surface tension forces arising during drying of the phage preparations in air.

Forces of surface tension evidently play a considerable part in the damage and deformation of phage particles. In this connection it is of interest that on moistening a dried phage preparation with a drop of water and drying it a second time the phage particles are damaged to such an extent that the outline of the heads becomes barely discernible. Tails remain in the field of vision, also considerably damaged (Fig. 3).

In order to discover a means of preventing damage to phage preparations, we decided to employ fixation and then follow the morphology of phage particles in relation to the action of various fixing agents.

Picric acid does not seem to exert appreciable fixing action on the phage particles, since the ratio between damaged particles and those of normal form was the same in such preparations as in the control, i.e., unfixed preparations.

1% trichloroacetic acid deforms phage particles and produces aggregation (Fig. 4).

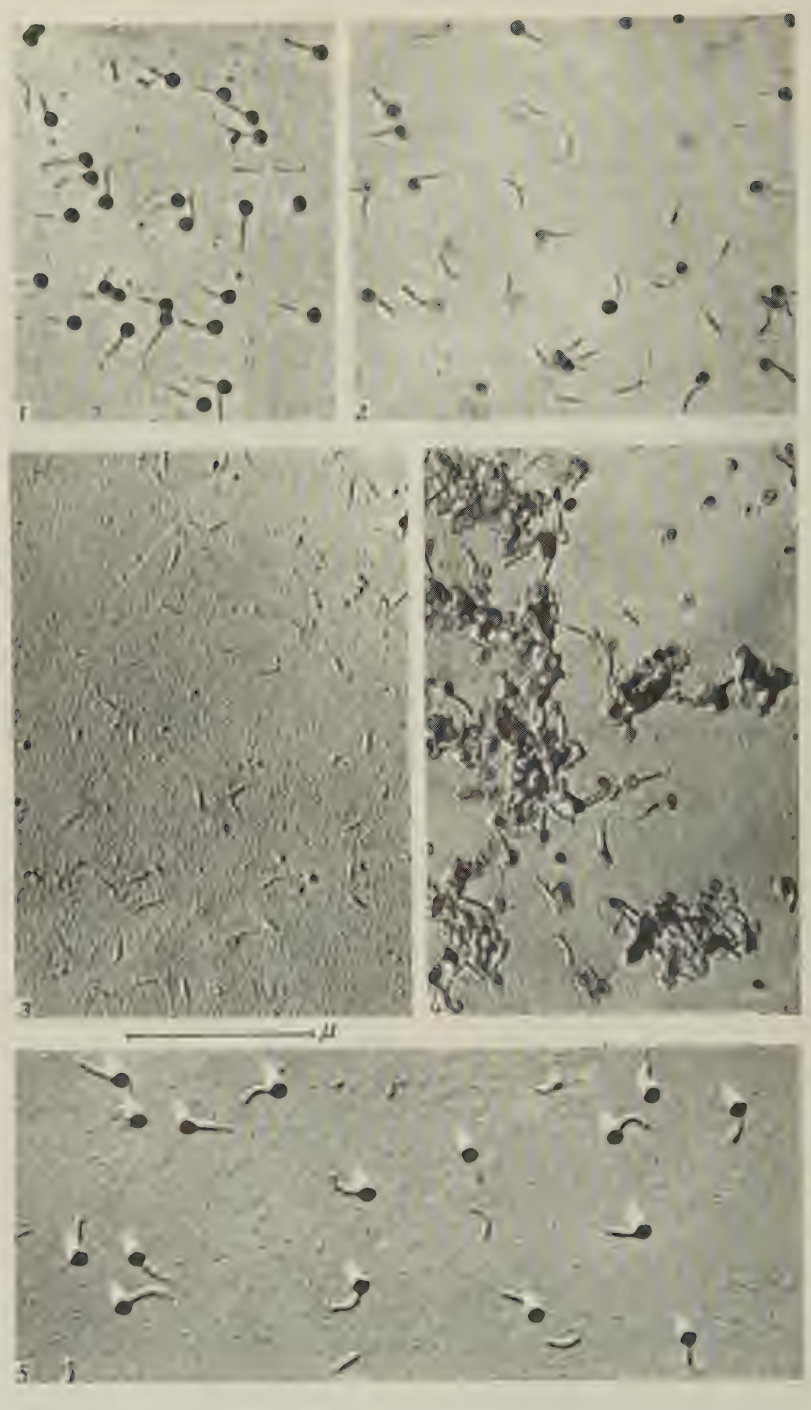

Fig. 1. Unfixed Bacillus mycoides phage. Fig. 2. Unfixed Bacillus mycoides phage.
Fig. 3. Unfixed preparation of Bacillus mycoides phage, dried and again moistened with a drop of water.
Fig. 4. Bacillus mycoides phage fixed by 1% trichloroacetic acid.
Fig. 5. Bacillus mycoides phage fixed by osmium vapors prior to dialysis for 10 min.

Fig. 6. Bacillus mycoides phage fixed by 1% buffer solution of OsO_4 for 15 min prior to dialysis.

Fig. 7. Bacillus mycoides phage fixed by formalin vapors for 30 min prior to dialysis.

Fig. 8. Bacillus mycoides phage fixed by formalin vapors for 30 min after dialysis.

Fig. 9. Bacillus mycoides phage fixed by formalin vapors for 24 hours with accompanying dialysis.

Fig. 10. Bacillus mycoides phage fixed by 4% solution of normal formalin for 5 days prior to dialysis.

Table 1. Titers of Bacillus mycoides Phage No. 1 Before and After Dialysis (according to Appel'man)

Date of phage obtained	11/14/51				11/28/51				12/7/51				12/12/51				12/15/51			
	before		after		before		after		before		after		before		after		before		after	
Dialysis																				
Duplicates	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Titer	10^8	10^8	10^8	10^8	10^7	10^7	10^7	10^7	10^7	10^8	10^8	10^7	10^{10}	10^{10}	10^{10}	10^{10}	10^9	10^9	10^9	10^9

Many workers regard osmium as a good fixing agent and it is used extensively to fix various biological objects. On fixing phage particles with osmium vapors (before and after dialysis) for 15–30 minutes, the phage heads are fixed quite well, but the tail part is evidently fixed incompletely, for very often its distal end appears denuded (Fig. 5). The same result occurs with 1% buffer solution of osmic acid when a broth phage lysate is fixed for 15 minutes before dialysis. While the heads of phage particles appear dense and mostly undamaged, the tail covering sometimes disintegrates into separate subunits of which it is apparently composed, at times denuding the tail core completely (Fig. 6). A longer contact, for 24 hours, of the broth phage lysate with buffered osmium solution produces even more damage to the covering of the phage tail; the tail core remains intact.

It was found on fixing phage particles with formalin that short-term contact of the phage lysate with the formalin before dialysis, either with vapors (up to 30 minutes) or 2–4% formalin solution for 1–2 hours, does not prevent damage to phage particles. On fixing with formalin vapors prior to dialysis for 15–30 minutes, in addition to normal phage particles there could be seen in the preparations particles with "shadows" instead of heads and tail cores denuded at the end (Fig. 7). But the same phage preparation fixed with formalin in vapors for 30 minutes, after dialysis, contained dense, compact heads and intact tails of uniform width throughout their length (Fig. 8). Phage particles remain intact to a greater extent when the broth phage lysate is fixed with 2–4% formalin solution for 1–2 hours with subsequent dialysis than when fixed with vapors before dialysis. Yet sometimes particles may be encountered in such preparations with depressed heads less dense to electrons and partially damaged tails.

Good results were obtained in fixation with formalin vapors continued throughout dialysis, and when the broth phage lysate was fixed with 4% formalin solution for 24, 48, 72 hours and even for 5 days with subsequent dialysis. Phage particles in such preparations had dense heads mostly hexagonal in shape, and straight undamaged tails (Figs. 9 and 10). It should be noted that tail rigidity is evidently increased by formalin, since in unfixed preparations or those fixed with formalin for a short time or with other fixing agents, the phage tails were generally curved, while after prolonged fixation with formalin the tail, as a rule, appeared straight.

Short-term fixation with formalin (both vapors and 2–4% formalin solution with subsequent dialysis) gives incomplete fixation of phage particles apparently by virtue of the fact that the formalin is readily washed out during dialysis and thus its fixing action is completely or partially eliminated. Therefore the phage particles, when the dialyzed preparation is dried, again become sensitive to the action occurring during drying of the preparation in air to a greater extent than phage particles fixed with formalin for a longer time or after dialysis. Similar facts were noted previously by Kriss and Biryuzova (1951), who employed formalin as fixing agent for bacterial cells.

Thus, the conclusion can be drawn from the material presented that particles of B. mycoides phage are in themselves very labile and readily deformed and damaged during drying of the preparation in air; this can be eliminated to a considerable extent by means of fixing the phage particles. The dialysis process, as is evident from the results presented, does not alter the phage's lytic property or damage the particles, since preparations fixed with formalin vapors after dialysis contained particles of normal appearance.

Of the fixing agents we studied, the best results were obtained in fixation with formalin vapors for 24 hours and with 2–4% formalin solution for 24 hours and longer. It should be noted that even 5-day formalin fixation did not damage phage particles; this makes it possible to use this fixing agent to preserve material over a long period of time.

As Staehelin (1958) has pointed out, when tobacco mosaic virus is treated with formalin, the latter reacts with the virus protein forming cross bonds; this makes the virus more stable to the damaging action of detergents and heat. Possibly the same mechanism operates in the fixation of phage particles with formalin, increasing their stability to the processes occurring during drying of preparations in air.

It was found in fixing phage particles with osmium that different parts of the phage corpuscle vary in their reaction to fixation. Whereas the phage head remains convex and dense, the tail covering not only is fixed inadequately but sometimes undergoes damage, consisting of its disintegration into separate, smaller elements. These facts compel the assumption that the nature of the phage corpuscle protein is not homogeneous. Therefore it is not unlikely that in fixing phage the use of a mixture of fixing agents that would produce fixation of the phage corpuscle as a whole would be advisable. This conclusion, in our opinion, can also be applied to more complex biological specimens.

SUMMARY

1. When purified preparations of Bacillus mycoides phage are dried in air, the phage particles are damaged

by surface tension forces with resultant great morphological diversity in the particles.

2. Formalin fixation (vapors and 4% solution) of phage preparations aids in preserving the normal structure of the phage corpuscle, i.e., dense, convex head of hexagonal shape and intact tail.

3. The varying degree of fixation by the same fixing agent in different parts of the phage corpuscle suggests that the protein of the phage membrane is not homogeneous.

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MYCORRHIZA FORMATION AND METHODS OF ITS STIMULATION IN LARCH IN THE TRANSVOLGA BLACK EARTH SOIL CONDITIONS

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Practice has shown that larch seedlings cannot be grown in any of the black earth soil varieties of the Transvolga region. In carbonate black earth soils the seeds are completely destroyed, in the other soil varieties the yield of seedlings per hectare is low and extremely variable, ranging from 14,000 to 235,000 per hectare with a possible yield of 1.5 million.

We already know from the literature data (Vekhov, 1949, Lisin, 1949) and our previous investigations (Tserling, 1958) that larch belongs to a species with a severely mycotrophic type of nutrition and that its cultivation without mycorrhiza is impossible.

The difficulties of cultivating larch seedlings in black earth soils are apparently due to the peculiarities of mycorrhiza formation on roots under these soil conditions.

Experimental cultivation of larch seedlings in carbonate black earth soils of the Transvolga region has shown that when larch is first sown, even artificial mycorrhization does not produce complete 100% infection of the seedlings. As a rule, only small single groups ("foci") of mycorrhized seedlings appear in the first year. These are immediately distinguishable by their better growth and green color of their cones from the stunted, morbid seedlings without mycorrhiza. In the second year there is a gradual proliferation of the foci, i.e., the mycorrhiza-forming fungus spreads to the hitherto unaffected seedlings adjoining the foci. These seedlings, in proportion to their infection by the mycorrhiza mycelium, begin to grow and their cones change color, nevertheless, in growth, they considerably lag behind previously infected seedlings. The earlier and quicker they proliferate and the individual foci merge, the better the effect on the general condition of the plantation and ultimately, on uniform growth and the total yield of standard seedlings.

Mycorrhiza-free seedlings begin to fall out already in the first year and by the end of the second year only isolated foci with mycorrhiza seedlings remain in the sown areas.

As part of the investigations now being carried out to elucidate the conditions of mycorrhiza formation in different areas of the Kuibyshev region, larch seed plantations in nursery gardens were examined. The other objects of the examination were the elucidation of the possibility of spontaneous mycorrhiza formation and the determination of the effect on mycorrhiza formation of soil conditions and repeated cultivation of seedlings in the same place. We examined seed plantations in carbonate black earth soils of the Transvolga region (Kinel'—SChI, Krotovsk nursery), in ordinary black earth soils (Koshkinsk nursery), in podzolized black earth soils (Krotovsk nursery of the Kamyshlinsk forest, Kuibyshev Region and the nursery of the Chulpanovsk forest of the Tatar ASSR). The results of the examination are presented in Table 1, from which it will be observed that the most unfavorable soil varieties for the cultivation of larch seedlings are the carbonate black earth soils.

Sowings made in 1955 in the carbonate black earth soils of the Krotovsk nursery, without the addition of mycorrhized soil, completely fell out. When mycorrhized soil was added and irrigation provided (Kinel'—SChI), by the second year only isolated foci with mycorrhizal seedlings remained.

In the Koshkinsk nursery (ordinary black earth soil) sowings were also made with the addition of mycorrhized soil but there was no irrigation. In the first year the sown area had a large number (as compared with sowings in carbonate black earth soils) of single foci with mycorrhiza seedlings among a mass of morbid, mycorrhiza-free seedlings which gradually fell out.

During the second year a number of the mycorrhiza foci merged, but not completely. As a result of the depletion, single foci frequently turned out to be at a distance of 1–5 m from each other. The long time it took for the seedlings to become infected with mycorrhiza also affected their development. The height of three-year seedlings varied within the range 15–90 cm.

From Table 1, it will be seen that there was no mycorrhization of the soil when sowings were made in podzolized black earth soils in the Krotovsk nursery and in the Kamyshlinsk and Chulpanovsk forest nurseries. Despite this, however, single foci with properly growing seedlings were recorded in the first year.

On examining, through a binocular microscope, the root system of seedlings taken from such foci, typical mycorrhiza envelopes on the absorptive roots were found (Fig. 1). This shows that it is possible for "spontaneous" (natural) infection of larch seedlings with mycorrhiza to occur under conditions of the podzolized black earth soils of the Transvolga region. This fact has a practical significance and is of interest in that the peculiarities of the ripening of larch seeds and their storage excludes the possibility of bringing in mycorrhiza-forming mycelium together with the seeds.

Table 1. Mycorrhiza Formation in Larch Seedlings in the Nurseries of the Transvolga Region

Investigated nurseries	Soil	pH of salt extract	Total alkalinity in %, HCO_3 to weight of salt extract of the soil	Mycorrhization	Provision of irrigation		Mode of propagation of mycorrhiza in one-year seedlings	Yield of seedlings in thousands per ha (in the second year)
					In first year	In second year		
Kinel', SKhI, sowings made in 1955 and 1957	Carbonate black earth	7.4	0.06	+	+	+	By isolated foci	40
Krotovsk 1955 sowing	The same	7.2	0.06	—	+	—	No mycorrhiza	100% fall out
Koshkinsk 1955 sowing	Ordinary black earth	6.4	0.03	+	—	—	Focal	235
Krotovsk 1956 sowing	Podzolized black earth soil	5.4	0.02	—	+	+	Focal	600
Kamyshlinsk forest, 1957 sowing	The same	5.4	0.018	—	—	—	Focal	Unknown
Chulpanovsk forest, 1957 sowing	The same	5.8	0.02	—	+	—	Focal	136

To elucidate the influence of soil conditions on the intensity of mycorrhiza formation in all the investigated areas, soil samples were taken for the determination of pH and total alkalinity.

From Table 1, it will be observed that if the administration of mycorrhiza soil to carbonate black earth soils (pH=7.4) fails to secure the merging of mycorrhiza foci in the second year even under conditions of irrigation, as a result of which the yield of seedlings remains extremely low, so a similar administration to ordinary black earth soil (pH= 6.4) produces a better mycorrhiza formation and a partial merging of the foci in the second year. When the acid reaction of the soil is greater pH= 5.4–5.8 (Krotovsk nursery and the Kamyshlinsk and Chulpanovsk forest nurseries) even "spontaneous" mycorrhiza formation is possible. This shows that there is a direct dependence between the speed of propagation of the mycorrhiza-forming mycelium on the one hand and the acidity of the soil on the other.

Thus, spontaneous infection and artificial mycorrhization produces the same picture in all the varieties of black earth soils—against a general background of mycorrhiza-free, morbid seedlings there is a number, greater or smaller, depending on the acidity and humidity of the soil, of single foci with mycorrhiza seedlings of normal growth.

The presence of mycorrhiza-free seedlings produces a falling out already in the first year of sowing and this becomes more acute in the second year. Consequently a complete merging of the foci does not occur and the yield of seedlings per hectare diminishes. Hence the decisive factors in the successful cultivation and production of a maximum yield of standard larch seedlings are those conditions which ensure a 100% infection—i.e., produce one continuous focus of mycorrhize seedlings in the first year of life.

The effect on mycorrhiza formation of repeated cultivation of larch seedlings in one and the same place was observed on the sowings in the Chulpanovsk forest nursery.* Here, seeds of one group were sown by identical agrotechnical procedures at one and the same time on three sites situated close to one another but previously used for the cultivation of different trees.

Site 1. Antecedent—apple tree. Larch was being cultivated here for the first time.

Site 2. Antecedent—larch. Larch was being cultivated here for the second time (following the digging up of second-year larch seedlings.)

Site 3. Antecedent—larch. Larch was cultivated here for the third time in succession.

At these sites, the intensity of mycorrhiza formation on larch shoots was affected differently by the different antecedents.

On the site where larch was being grown for the first time, mycorrhiza seedlings had single sparse foci during the first year. On sites used for repeated larch growing, closely bound seedlings of equal height completely covered the sown areas, i.e., presented, as it were, a complete area of mycorrhiza seedlings. Moreover, on the site where larch was being grown for the third time, the seedlings were higher. The effect of repeated cultivation of larch in the same place which was already apparent in the first year was even more pronounced in the second year.

On the site where larch was being grown for the first time, sparse mycorrhiza foci developed very slowly and did not merge, nor was there any mass mycorrhiza infection of seedlings. As a result the mycorrhiza-free seedlings fell out during the second year and only single foci with mycorrhiza seedlings remained in the sown area (Fig. 2). On the two adjoining sites where the shoots became infected with mycorrhiza during the first year, the seedlings did not fall out and their better development was visible to the naked eye (Figs. 2 and 3). If the site where larch was being grown for the first time is taken as control, a comparison with the two adjoining sections gives the results presented in Table 2.

On the site where larch was being grown in a given spot for the second time, the number of seedlings was

*The Chulpanovsk forest adjoins the northern border of the Kuibyshev region.

Table 2. Effectiveness of Repeated Cultivation of Larch Seedlings on the Same Place

Test versions	Height of seedlings in cm M± m	Differs from control D ± m_d	Reliability D: m_d	Yield of seedlings in thousands per hectare
Larch being grown for the first time in a given area	12.4±0.41	—	—	136
Repeated larch cultivation .	27.0±0.60	14.6±0.73	20	1250
Larch being grown for the third time in a given area	31.0±0.53	18.6±0.67	27	1590

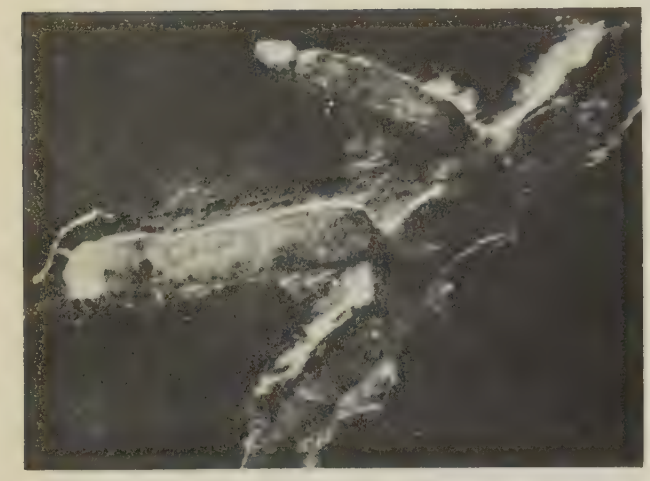

Fig. 1. Spontaneously produced mycorrhiza sheath covering absorptive roots of larch.

Fig. 2. In the foreground, site where larch was being grown for the first time.

Fig. 3. Site where larch was being grown for the third time in succession.

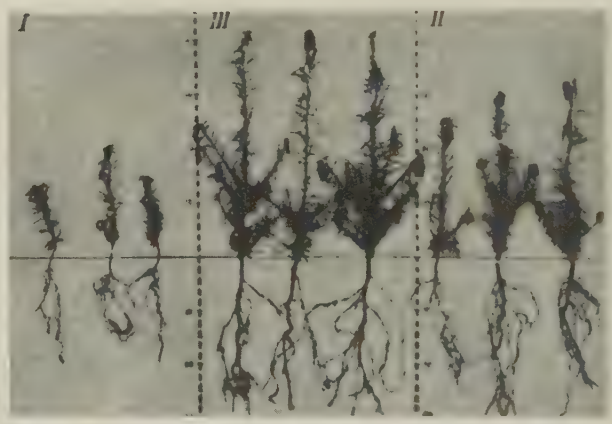

Fig. 4. Effect of repeated cultivation in the same place on the development of seedlings: 1) Larch grown for the first time; 2) for the second time; 3) for the third time.

almost ten times greater and the average height of the seedlings was twice that of the control.

On the site where larch was being grown for the third time, the number of seedlings was 11.5 times greater than that of the control and the average height of seedlings was 2.5 times greater (Fig. 4). It was interesting to compare the effectiveness of spontaneous mycorrhization in leached black earth soil of the Chulpanovsk forest nursery with the spontaneous mycorrhization in the same kind of soils but under the better conditions of moisture in the Krotovsk nursery. In the Chulpanovsk forest nursery the larch seedlings were watered only four times during the summer of the first year; in the Krotovsk nursery watering was more frequent and not only in the first but in the second year as well.

Because of this, in the Krotovsk nursery during the second year many of the mycorrhiza foci merged and the yield of seedlings was 600,000 per hectare as against 136,000 from the first-year growth in the Chulpanovsk forest nursery.

A different picture appears when we compare these plantations of the Krotovsk nursery with repeated cultivation on the same site in the Chulpanovsk forest nursery. Given the same conditions of moisture the yield of seedlings per hectare was twice as high in the Chulpanovsk forest nursery; 1,250,000 as against 600,000 in the Krotovsk nursery.

The leached black earth soils of the Transvolga region apparently contain a mycorrhiza forming fungus but in an amount insufficient to promote mass mycorrhiza formation in the seedlings during the first year even in conditions of adequate moisture.

Thus, repeated cultivation of larch in the same place enriches the steppe soils with mycorrhiza-forming fungus, excludes the presence of seedlings which fail to develop and prevents their fall.

In 1958, in order to verify the results already obtained, we once more repeatedly cultivated larch on one site in the Chulpanovsk forest nursery. The plantation in the area where larch had not previously been grown—antecedent—ash—served as control. After repeated cultivation, a mass (100%) mycorrhiza formation in seedlings was achieved in the first year whereas in the area where it was being grown for the first time, mycorrhiza seedlings formed small foci. This once more confirmed the effectiveness of repeated cultivation of larch on the same site.

SUMMARY

1. Natural (spontaneous) mycorrhiza formation on the roots of larch seedlings is possible in the acid varieties of black earth soils of the Transvolga region (pH = 5.4–5.8).

2. The low and extremely variable yield of larch seedlings per hectare in the nursery gardens of the region is due to lack of mycorrhiza formation in the first year. Both upon spontaneous infection and artificial mycorrhization only single groups (foci) with properly growing mycorrhiza seedlings appear. The main bulk consists of morbid, mycorrhiza-free seedlings which soon stop growing and gradually fall out.

3. Natural mycorrhiza formation and the effectiveness of artificial mycorrhization depend upon the acidity and humidity of the soil. Both in cases of moisture deficiency and alkaline reaction of the soil solution the mycelium of the mycorrhiza-forming fungus spreads slowly and does not promote mass infection of the seedlings.

4. Administration of mycorrhiza soil is a prerequisite for the primary cultivation of larch in a given area. Moreover, in carbonate black earth soils (because of their alkaline reaction) it is necessary to apply suitable agrotechnical measures which improve mycorrhiza formation, in particular those which increase the acidity of the soil and pollution. Repeated sowing in the same soil obviates the necessity for additional mycorrhization.

5. In order to enrich steppe soils with mycorrhiza-forming mycelium it is necessary to grow larch seedlings in nurseries on a separate plot without changing the culture over a period of years. The cultivation of larch in the same place is the most perfect method of mycorrhization of steppe soils. In this way it is possible to reduce to a minimum the expenditure of labor, funds and time, and to ensure 100% mycorrhization of one-year seedlings. By repeated cultivation it is further possible to increase the yield of seedlings per hectare by 10–11.5 times and to increase their height by 2–2.5 times.

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OCCURRENCE OF SULFATE-REDUCING ORGANISMS IN OIL-BEARING FORMATIONS OF THE KUIBYSHEV REGION WITH REFERENCE TO SALT COMPOSITION OF LAYER WATERS

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Among the microorganisms found in layer waters of oil-bearing formations, sulfate-reducing bacteria occupy an important place.

Many investigators have already provided a good deal of information on the occurrence of this group of microorganisms. Ginsburg-Karagiccheva (1926), Shturm (1951), Tauson and Aleshina (1932), Ekzertsev and Kuznetsov (1954) have demonstrated that sulfate-reducing organisms are present in oil-bearing rocks. Kolesnik (1955), Simakova and Lomova (1958) have stressed the fact that in Devonian layer waters, sulfate-reducing organisms are found very rarely, and that these strains have a low activity. They considered this to be due to the fact that Devonian layer waters are not a favorable medium for the development of sulfate-reducing and other organisms as they contain high concentrations of chlorides of calcium and magnesium and practically no sulfates and phosphates, and because the waters give a very acid reaction. However, this seemed to us an inadequate explanation, as in many Devonian layer waters, the content of the SO_4 ion is 70–80 mg/l. Such a quantity of sulfates is undoubtedly sufficient for utilization by the organisms.

Secondly, the absence of phosphates is also not an adequate reason, as there are Carboniferous and Permian waters in which the phosphates are also absent, yet in these waters different groups of microorganisms including sulfate-reducing organisms have been found.

Thirdly, it is not always possible to explain the absence of sulfate-reducing organisms by the acid reaction of the medium, as certain Devonian layer waters are not very acid: pH = 6.0–6.5. Such are the deposits Zhigulevsk in the layer D-O and the Zol'nyi gully in the layer D-1 (Table 1).

Table 1. Effect of pH on the Development of Sulfate-Reducing Organisms in Distilled Layer Water from D-II Zhigulev Oil Deposit

Water %		Increase in H_2S after 168 hr at 27° (mg/l)	
layer	fresh	pH = 5.75	pH = 7.2
100	0	0	0
50	50	0	0
58	42	0	0
66	34	42	7
72	28	73	50
77	23	89	50
83	17	147	125
89	11	224	160

According to the data of many investigators, a medium which gives such a reaction is quite suitable for the development of sulfate-reducing organisms (Zobell, 1958). Moreover, there are a number of deposits in Kungur where the layer waters have a pH of 5.8–6.2 and the same high mineralization as in Devonian waters even though the hydrogen sulfide content in these waters is 600–700 mg/l (Belyakova). It also seems unclear why it is not possible to promote the development of the sulfate-reducing bacterial strain in layer waters of Devonian deposits by introducing the requisite nutrient substances when these organisms develop well at the natural outlets to the surfaces of the earth (sources of the Shorkin gully in the Kuibyshev region).

In view of all this, and also because sulfate-reducing organisms play such an important role in the life of oil deposits, we thought it of interest to elucidate the factors which restrict and stimulate the development of these organisms in oil formations.

We therefore investigated the oil deposits of the Ural-Volga oil-bearing region. Table 2 shows the occurrence of sulfate-reducing organisms in layer waters, determined by inoculation on a Tauson agar medium in the 1950 Shturm modification.

In the table the mineralization of layer waters is shown in mg/equiv. per 100 g of water. The table also gives the hydrogen sulfide contents in the waters and the correlation between the content in mg/equiv of divalent and monovalent ions. These correlations were calculated from the analytic data provided by the workers of the State Institute for the Design and Planning of Petroleum Industry Establishments in the Eastern Regions (GIPROVOSTOKNEFT') in Kuibyshev. Most of the data on the content of hydrogen sulfide in layer waters was supplied by A. N. Kozin.

Table 2 shows that in practically all the specimens of Permian layer waters, sulfate-reducing organisms were present in tens of thousands per 1 ml of water. A somewhat smaller number was found in layer waters of Carboniferous deposits but not in the layer waters of the fourth stratum of the coal-bearing level of the Mukhanov oil deposit. In these waters hydrogen sulfide was also absent.

Lower down along the section, sulfate-reducing organisms were not found in Devonian layer waters except in a few specimens.

Table 2. Occurrence of Sulfate-Reducing Organisms in Oil Deposits of the Kuibyshev Region and the Chemical Composition of Layer Waters

Deposit	Tested stratigraphic level	Bacteriological analysis. No. of water specimens causing devel. in Tauson medium					Results of chemical analysis		
		No. analyzed	No. identified as positive	with lactate Ca	with acetate Ca	with oil	total mineralization, mg/equiv/100 g water	$\frac{Ca^{++} + Mg^{++}}{K^{+} + Na}$	H_2S, mg/l
Kalinovsk	Permian KS	61	56	51	50	56	205,5—445.7	0.03—0.11	255—1975*
Pokrovskoe	A_3	2	2	2	2	—	43.72—289.26	0.22—0.26	63.5—379.0
Sernovodsk	B_2	3	3	3	2	1	724.20—755.52	0.18—0.24	Present
Syzran	B_1	3	2	1	1	1	404.92—419.26	0.35—0.36	Present
	B_2	3	2	1	0	2	390.06—406.38	0.35—0.36	214.0—422.0
Gubinsk	B_2	3	3	1	2	2	503.86—519.48	0.32—0.33	135.0—264.0
Yablonovo gully	B_0	1	1	1	0	0	—	—	Есть
	B_2	1	0	0	0	0	—	—	80.0—181.0
Strel'nyi gully	B_2	2	1	1	0	1	716,68	0.15	Есть
Zol'nyi gully	B_2	8	8	5	5	3	741.16—797.32	0.16—0.23	195.0—434.0
Mukhanov	Kungur	2	2	1	1	2	363.04—800.0	0.07—0.08	18.4—518.0
	I layer C_1	9	8	4	4	5	823.84	0.06	308.9*
	I + IV layer C_1	1	1	1	0	0	825.40	0.06	648.9*
	IV layer C_1	5	0	0	0	0	711.48—796.06	0.40—0.44	0
	D-II	1	0	0	0	0	824.04	0.73	0
	D-II + DIII	3	0	0	0	0	837.02	0.98	0
	D-III	1	0	0	0	0	840.44	0.98	0
Yablonovo gully	D-II	2	1	0	1	0	768.40	0.38	0
	D-III	2	1	0	1	0	761.62	0.30	0
Zhigulev	D-0	2	0	0	0	0	—	—	0
	D-II	4	0	0	0	0	—	—	0
	D-III	0	0	0	0	0	—	—	0
Zol'nyi gully	D-I	6	3	2	1	1	820.28	0.75	0
	D-II	2	2	1	1	0	813.08—818.64	0.57—0.68	0
Sources at the Shorkin gully	—	1	1	1	1	1	270.5	0.75	Present

*The determinations are ours. The data of the chemical analyses were provided by the Candidate of Chemical Sciences A. N. Kozin (State Institute for the Design and Planning of Petroleum Industry Establishments in the Eastern Regions) (GIPROVOSTOKNEFT').

Table 3. The Oxidizing-Reducing Conditions in Layer Waters of Oil Wells in the Kuibyshev Region

Deposit	Well No.	Level worked	Method of extraction	Eh, mV	pH	rH_2	Fe^{+++}, mg/l	Fe^{++}, mg/l	H_2S, mg/l
Mukhanov	57	I layer C_1	Sludge pump	−202	6.2	5.5	—	—	308.9
" "	19	IV layer C_1	" "	— 15	5.5	10.5	0.48	181.0	0
" "	18	D-II	" "	+ 10	5.6	11.6	0.18	530.0	0
" "	50	D-II and DIII	" "	+ 73	5.64	13.9	0.8	80.0	0
" "	400	D-III	" "	— 6	5.7	11.2	0.8	80.0	0
Zol'nyi gully	1	b_2	Gauge cock	−118	6.7	9.4	—	—	Present
" "	7	"	" "	−123	6.9	9.6	—	—	"
" "	68	"	" "	−150	7.0	8.9	0	0	"
" "	54	D-I	" "	+ 15	6.5	12.5	0.4	99.5	0
" "	102	"	" "	— 10	5.75	11.2	0.25	277.0	0
" "	79	D-II	" "	+ 3	5.8	11.7	0.4	180.0	0
Zhigulev	63	"	" "	+ 20	5.6	11.9	0.25	92.0	0
Kalinovsk	286	KC	" "	−205	7.1	6.9	0	252.0	1975.6
"	509	"	" "	−194	7.3	7.7	0	80.0	684.6

In these specimens the organisms had a low activity. Thus our findings agree with the data obtained by Kolesnik (1955) and Simakova and Lomova (1958). To elucidate the factors which restrict and stimulate the development of sulfate-reducing bacteria, we carried out several investigations.

Tests were made to determine to what extent the acid reaction of the medium inhibits the development of sulfate-reducing organisms. For this, layer water taken from D-II Zhigulev oil deposit was neutralized by caustic soda to pH = 7.2 and diluted in various proportions with fresh water. The rest of the water was similarly diluted but the pH was left at 5.75, i.e., the natural level. To each dilution the requisite nutrient salts and sulfate-reducing bacterial culture were added. As will be seen from Table 1, sulfate-reducing organisms developed in dilutions consisting of 34% layer water and 66% fresh water. This shows that an acid reaction of 5.75 in the medium does not inhibit the development of sulfate-reducing organisms. From this it may also be concluded that distillation of layer waters considerably intensifies the action of the organisms, although high mineralization does not in itself preclude the development of sulfate-reducing organisms in layer waters. These microorganisms, together with large concentrations of hydrogen sulfide, are found in highly mineralized waters the specific gravity of which is 1.17–1.19 (first layer, C_1 Mukhanov deposit: Table 2). According to Belyakova (1956) there is up to 600–700 mg/l of hydrogen sulfide in some of the highly mineralized Permian waters.

Temperature, which in the Devonian layers of the Kuibyshev region reaches 71°, also does not prevent the development of sulfate-reducing organisms. Strains are known which develop well at 55–65° at normal pressure. On increasing the pressure to 1000 atm the organisms can develop at 140°C (Zobell, 1958). Kuznetsov (1955) found sulfate-reducing organisms in the thermal sources of Kamchatka where the temperature is 55–70°.

Our measurements of the oxidizing-reducing potential in layer waters (Table 3) show that the Devonian layer waters differ significantly in this respect from the Carboniferous and Permian waters. Nevertheless, the absence of sulfate-reducing organisms in Devonian oil deposits cannot be explained in terms of the oxidizing-reducing potential as the organisms can develop within the limits of the magnitudes found for Eh.

The Eh of layer waters from Carboniferous deposits ranged from –118 to –202 mv. Eh of layer waters from Permian deposits ranged from –194 to –205 mv,

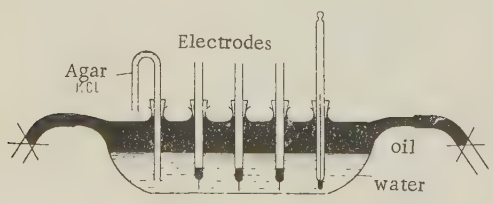

Fig. 1. Vessel for determining Eh in layer waters.

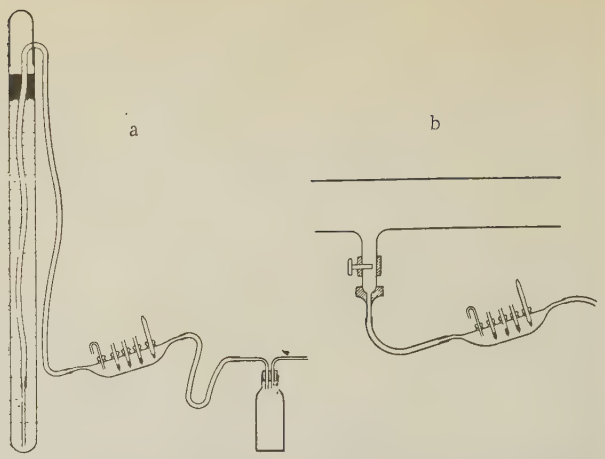

Fig. 2. Plan of extraction of water specimens from (a) sludge pump (black patch indicates layer of oil) and from gauge cock (b) for the measurement of Eh.

Table 3. The Eh was determined in a special sealed vessel with three electrodes (Figs. 1 and 2). In extracting specimens care had to be exercised that no air was admitted. It was found that sulfate-reducing organisms could develop at all the determined Eh values. Yet they do not develop in the Devonian waters. Even the administration of all the requisite nutrient salts and the adjustment of pH to neutral (Table 1) failed to promote their development in these waters. What then is the factor that plays such a decisive role in the inhibition of the development of sulfate-reducing organisms in Devonian layer waters?

An examination of the data presented in Table 2 shows that sulfate-reducing organisms develop in those layer waters in which the relation of the sum of Ca and Mg in mg equiv to the sum of Na and K in mg equiv is less than 0.36–0.40. Waters in which the content of divalent ions is higher than that given above do not contain sulfate-reducing organisms.

The few exceptions to this rule seen in Table 2 can be explained by the flow of Carboniferous waters into the Devonian deposits (Yablonovo gully, Zol'nyi gully) or by the considerable dilution of the water on the earth surface (sources at the Shorkin gully).

As our investigations have demonstrated (Table 1), a threefold dilution of layer water from the Devonian deposits with fresh water renders it suitable for the development of sulfate-reducing organisms. Experience in the flooding of oil wells has shown that when the Devonian layer waters are thoroughly distilled (layer D-1 Romashkin deposit in Tatar SSR), the sulfate-reducing process in the oil deposits begins (Glumov and Stankevich, 1959).

From all this it would appear that the main factor restricting the development of sulfate-reducing organisms in layer waters of Devonian oil deposits is the preponderance of calcium and magnesium over sodium and potassium when the over-all mineralization of the waters is 700–800 mg/equiv of salts. When the layer waters are thoroughly diluted with fresh waters the harmful effect of calcium and magnesium loses its force and the development of sulfate-reducing organisms becomes possible. This assumption is supported by the results of laboratory tests (Fig. 3).

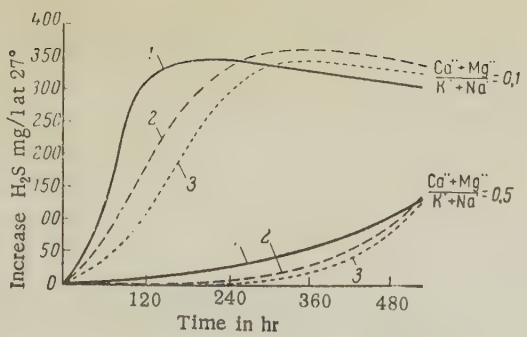

Fig. 3. Effect of the positively-charged ion content of the medium on the development of sulfate-reducing organisms. 1) 5.5% Cl^-; 2) 6.1% Cl^-; 3) 6.7% Cl^-.

This test shows that a correlation of 0.1 between the sum of divalent and monovalent + charged ions favors the development of sulfate-reducing organisms better than a correlation of 0.5 even when there is such a development—favoring salt content as 100–140 g/l. The data in Table 1 show that the border which divides hydrogen sulfide-containing waters from those which do not contain it in the oil deposits of Kuibyshev region lies between the Carboniferous and Devonian layers whereas in the Mukhanov deposit it runs between the first and fourth layers of the coal-bearing level (we had no specimens from the second and third layers). The role of individual positive ions of layer waters and the preponderance of Cl^- ions over HCO_3^- ions in the suppression of the development of sulfate-reducing organisms remains, for the present, unsolved.

In conclusion I should like to express my sincere thanks to the Candidate of Chemical Sciences A. N. Kozin—Head of the Department of Oil Drilling and Geology of the State Institute for the Design and Planning of Petroleum Industry Establishments in the Eastern Regions—and to all workers of petroleum establishments for their active cooperation.

SUMMARY

1. The principal factor restricting the development of sulfate-reducing organisms in Devonian layer waters is the preponderance of divalent ions over monovalent ones when the total mineralization is 700–840 mg/equiv. of salts per 100 g of water. Other factors play a secondary role and only intensify the unfavorable effect of divalent ions.

2. The ratio between the sum of mg equiv. Ca^{++} and Mg^{++} and the sum of mg/equiv Na^+ and K^+ we have designated the "cation coefficient".

3. When mineralization of layer waters is 260–840 mg equiv of salts, the "cation coefficient" 0.36–0.4 becomes critical. At higher values sulfate-reducing organisms do not develop in the layer waters, at lower values they do.

4. It may be assumed that when mineralization is less than 260 mg/equiv the harmful effect of divalent ions disappears. Thus, development of sulfate-reducing organisms can be achieved by diluting Devonian waters with fresh waters.

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A COMPARATIVE STUDY OF NAPTHALENE-OXIDIZING ORGANISMS IN UNDERGROUND WATERS

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For many microorganisms oil hydrocarbons are easily available and valuable energy substances.

At present over 100 species of microorganisms are known which are capable of oxidizing oil, wax, paraffin and individual hydrocarbons of different homologic ranges (Birshtreher 1957). The study of microorganisms which decompose hydrocarbons is of great importance for the solution of a number of practical problems such as prospecting for oil and gas, the purification of sewer waters from chemically resistant compounds and so on; moreover, it can throw light on the question of the origin and destruction of oil.

In the literature, a number of naphthalene-oxidizing organisms are described. Tauson (1929, 1950) devoted to naphthalene-oxidizing organisms a special study in which he described three species isolated from the soils of Baku and Transcaucasian oil wells. He also developed methods of isolating and cultivating naphthalene-oxidizing organisms and studied the chemism of oxidation. Gray and Thornton (1928) found naphthalene-oxidizing organisms in arable and wild soils of the Rothamstead experimental station and demonstrated that naphthalene introduced into the soil is destroyed mainly by the action of these organisms. Erikson in 1957 found that of the nine varieties of microspores which he isolated from lake mud five could oxidize naphthalene. In America a number of papers have been published on the chemism of naphthalene oxidation and on the conditions promoting this oxidation. The naphthalene-oxidizing microorganisms described in these works were isolated from the soil. In most cases the soil was first "enriched" with the appropriate microorganisms; it was mixed with sterile naphthalene, moistened and in that state left for several weeks. Stravinskii and Stone (1943, 1954) and Klausmeier and Stravinskii (1957) isolated in this way Pseudomonas aeruginosa. The microorganisms obtained by Murphy and Stone (1955) are very similar to Pseudomonas arvilla and Pseudomonas rathonis (for purposes of identification the authors used the Bergy determinator). An unidentified organism isolated by Walker and Wiltshire (1953) resembles Tauson's Bact. nonliquefaciens. In the course of their investigations of the oxidation of naphthalene by these organisms, Murphy and Stone (1955), Stravinskii and Stone (1943, 1954), and Walker and Wiltshire (1953) found that salicylic acid is an intermediate product of this oxidation. Walker and Wiltshire (1953) claimed that D-trans-1,2-dihydro-1,2-dihydroxynaphthalene is also an intermediate product of naphthalene oxidation. Murphy and Stone (1955) isolated from the culture medium of P. aeruginosa a substance which they identified as 1,2-naphthoxynone. They regard this as a product of naphthalene oxidation which is not subject to further conversions but which accumulates in the medium and gives it a reddish-orange color. Of the conditions which favor naphthalene oxidation different authors mention aeration, a comparatively high pH level (8) and the presence in the medium of ions $Fe^{\cdot\cdot}$, $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Cu^{\cdot\cdot}$.

Between 1956–1959 we worked on the isolation of naphthalene-oxidizing organisms from underground waters of both oil and non-oil bearing regions of the USSR. The object of this was to enable us to carry out a comparative study of the spread of the organisms and to isolate, if such exist, groups or species specific to oil deposits.

For the experimental cultivation of naphthalene-oxidizing organisms we employed Tauson's method. To the mineral medium, naphthalene was added as the only source of carbon and energy. In the case of liquid cultures, atomized naphthalene crystals, sublimed in sterile conditions, were added directly to the medium. When inoculated on agar the volatilizable naphthalene settled on the surface of plates with mineral agar. The tests were carried out 28° in Erlenmeyer flasks with a capacity of 50 ml. Accumulating strains of naphthalene-oxidizing organisms were obtained by inoculating the waters under investigation into a liquid medium with naphthalene.

We investigated the underground waters of oil-bearing regions of eastern Georgia, western Turkmenistan and of the Grozno-Dagestan oil- and gas-bearing region as well as the waters of oil-bearing carboniferous deposits of the Moscow artesian basin. Specimens for analyses were taken from underground waters in different regions: (1) from sources of water-supplying levels, (2) from oil wells and prospecting areas and (3) from mineral sources where the underground waters are discharged. In all, 174 specimens were taken and studied.

In the Grozno-Dagestan oil- and gas-bearing region, in eastern Georgia and western Turkmenistan, naphthalene-oxidizing organisms were found only in water specimens from oil wells but not in any of the specimens from the regions of water supply and discharge. Moreover, in specimens of oil well waters the naphthalene-oxidizing organisms, as a rule, developed

actively and this development was accompanied by a number of characteristic features: the formation in the medium of a greenish-yellow pigment which subsequently turned brown, the "eating up" of naphthalene and the formation of a film or precipitate. Old cultures of naphthalene-oxidizing organisms which had accumulated over a period of two to three weeks were, as a rule, dark brown in color and formed a flaky precipitate at the bottom of the retort. In the Moscow artesian basin, on the other hand, inoculations on the same medium produced a slight turbidity and naphthalene from these cultures disappeared more slowly.

To obtain pure strains of naphthalene-oxidizing organisms, accumulated cultures were inoculated on meat-peptone and mineral agar with naphthalene; from these an inoculation (from one colony) was again made on a liquid medium. In all, five species of naphthalene-oxidizing organisms were isolated from the investigated waters. They were all gram-negative, mobile, spore-free rods. Two species actively oxidized naphthalene and formed a greenish-yellow pigment, a film and precipitate in the medium. All these features were less marked in a pure culture than in an accumulated one. In this respect, Tauson was right in maintaining that many microorganisms develop better in an accumulated culture than in a pure one, because in it the nutrient substances decompose gradually under the action of a few organisms, whereas in a pure culture, one organism can oxidize to any intermediate product which, on accumulating, either retards or completely inhibits development.

To identify the isolated species we used the Krasil'nikov determinator (1949).

Of the two species, one was identified as a variant of Pseudomonas scissa and was distinguished from it by its action on milk and starch; and the other one was a variant of P. pelliculosa (a variety of Pseudomonas fluorescens). The Krasil'nikov determinator does not indicate how P. scissa and P. pelliculosa act on naphthalene. Both these species were found only in specimens of underground waters from oil fields. The only exception was the specimen from the Moscow artesian basin which was taken from the MTS well in the village Mednoe where pollution by oil products is quite likely to occur. The variant P. scissa is fairly widespread. It was isolated as a pure strain from three water specimens from the Grozno-Dagestan oil- and gas-bearing region and from three specimens from eastern Georgia. The characteristic culture features, namely, the formation of a greenish-yellow pigment and film as well as the flat, folded and flower-shaped colonies of this species on a meat-peptone agar, made it possible to detect the organism in underground waters of other oil fields (Table 1). The variant P. pelliculosa was isolated as a pure strain from three specimens obtained from fields in eastern Georgia: the Norio field (well No. 26) and the Mirzaani field (wells 113 and 168).

Three other species which we isolated were less active in relation to naphthalene and developed only in a liquid medium but not on agar with naphthalene whereas the above-mentioned two species, on a mineral agar with naphthalene, formed colonies surrounded by an area of dissolved naphthalene. The three species were identified as variants of Pseudomonas pictorum, Pseudomonas desmoliticum and Bact. jophagum. The P. pictorum described by Gray and Thornton (1928) oxidizes phenol; our variant oxidizes naphthalene as well. In all other respects they were identical.

Table 1. Occurrence of the Variant Pseudomonas scissa in Underground Waters of Oil Fields

District	Oil field	Well No.	District	Oil field	Well No.
Grozno-Dagestan oil--gas bearing region	Novogroznenskii	6	Eastern Georgia	Norio	17
	"	33*		"	23
	Goragorskii	244		"	26
	Suvorov	72		"	31*
	Dag-Oni	19		"	57
	Malgobek	50/1		"	60*
	Makhachkala	—		Mirzaani	76
	"	46*		"	102*
	Ternair	9		"	168
	"	10			
	"	79*			

*From these specimens pure strains were obtained.

Table 2. Distribution of Variants Pseudomonas pictorum, Pseudomonas desmoliticum and Bact. jophagum

Organism	Place from which specimen was taken
Variant P. pictorum	Malgobek oil field—well 50/1*
	Ternair oil field—well 79
	Orekhovo-Zuevo, Moscow*
Variant P. desmoliticum	Makhachkala*
	Kalinin
Variant Bact. jophagum	Novogroznensk oil field, well 33
	Goragorsk oil field, well 244*
	Mednoe village MTS, Kovrov, Orekhovo-Zuevo, Moscow*

*From these specimens pure strains were obtained.

The variant P. desmoliticum does not grow on phenol but that described by Gray and Thornton does. The variant B. jophagum differs from the Gray and Thornton's B. jophagum by its action on milk and starch. The variants P. pictorum, P. desmoliticum, and B. jophagum are found in waters of oil-bearing as well as non-oil-bearing districts. They were found in the Grozno-Dagestan oil and gas bearing region and in the Moscow artesian basin (Table 2).

Accumulating cultures of naphthalene-oxidizing organisms were also obtained from waters from a number of oil fields in western Turkmenistan. Pure strains, however, could not be obtained as in the process of isolation they became attenuated and disappeared.

Finally, I should like to express my deep gratitude to the senior scientific worker Z. I. Kuznetsova and to the senior engineer V. M. Shvets for their help in this study.

SUMMARY

1. Five species of naphthalene-oxidizing organisms were isolated from underground waters of the Grozno-Dagestan oil-gas bearing region, from oil fields of the Georgian SSR and from underground waters of the Moscow Artesian basin.

2. Two species, the variant Pseudomonas scissa and the variant Pseudomonas pelliculosa, are specific to underground waters of oil fields.

3. The species of naphthalene-oxidizing organisms which are specific to underground waters of oil fields are the most active. Characteristic features of their development on a naphthalene-containing medium are the release into the medium of a greenish-yellow (browning) pigment and the formation of a film and precipitate.

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REDUCTION OF SULFATES BY SULFATE-REDUCING ORGANISMS IN MIXTURES OF SEA AND LAYER WATERS IN A MOLECULAR HYDROGEN ATMOSPHERE

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When oil beds are flooded with sea water to increase output under the secondary means of oil extraction, in a number of cases, hydrogen sulfide has been observed to form in the oil beds as a result of the reduction of sea water sulfates.

It is known that molecular hydrogen is formed in vast quantities as a result of geochemical, physicochemical, biochemical and other processes which occur both in the depths of the earth's crust and in the biosphere. Yet it is very rarely found in the composition of natural gases. Buneev and Kharitonova (1948), Shturm (1957), Sorokina (1957) Wight and Starkey (1945), Butlin and Adams (1947), and Zobell (1947) have demonstrated that sulfate-reducing organisms are capable of reducing sulfates to hydrogen sulfide in a molecular hydrogen atmosphere.

Bunker (1939), Wight and Starkey (1945) have demonstrated that sulfate reducing organisms can likewise use hydrogen formed in vitro in electrolytic corrosion of metallic iron. As a result of this process, the metal is subjected to anaerobic corrosion and becomes covered with a crust consisting of iron hydroxide and a graphite-like substance with admixtures of iron sulfide under it (Shturm, 1957).

Nevertheless, the process of hydrogen sulfate reduction in the conditions of the Baku oil installations has not yet been investigated. The object of the present work was the study of hydrogen sulfate reduction in mixtures of sea and different fresh waters by sulfate-reducing organisms. The ultimate object of this investigation is to develop measures against the hydrogen sulfide corrosion of oil equipment.

In the tests designed to study hydrogen sulfate reduction, layer water taken from the oil deposits under the Kirmakinsk formations of Artema island was used.

For the first series of tests water from well No. 234 (and for the second series water from well No. 366) was used. These were mixed with Caspian sea water taken from the region of the Buzovny village. The first series of tests was carried out in two variations; in the first a mixture consisting of 20% sea and 80% layer water was used and in the second variation the waters were mixed in different proportions.

To each mixture microscopic quantities of iron sulfate were added as an indicator and 1 ml of accumulating cultures of sulfate-reducing organisms. Five-hundred ml of this mixture was poured into retorts 1.5 liters in volume and air was pumped out by vacuum pumps. The retorts were then filled with molecular hydrogen. This procedure was repeated three times.

From the control retorts air was also pumped out and the retorts were hermetically sealed. The tests were conducted in a thermostat at 28°.

On the third day, a light grey film appeared on the surface of the liquid in the test retorts. After seven days both the film and the precipitate at the bottom of the retort turned black. In the control retorts no changes were observed. The water was chemically analyzed both before the commencement as well as during the development of the sulfate-reducing process.

Results of the analyses are presented in Table 1.

Results in Table 1 show that the initial quantity of sulfates in the first test variation was (20 : 80) 534 mg/l and that after 168 days it decreased to 312 mg/l.

After 237 days there was a further decrease to 252 mg/l and after 338 days to 196 mg/l. At the same time 128 mg/l of hydrogen sulfide was formed. The coefficients of initial alkalinity rose from 1.52 to 3.18%.

For the second series of tests we altered the conditions as follows: layer and sea water were mixed in proportions of 3 : 7 and 1 : 1, the retorts were filled to the brim and hydrogen was forced into them to squeeze the water out to the required level. The retorts were then hermetically sealed and placed in a thermostat at 28°. The process of sulfate reduction in this series of tests began on the third day; this was clearly indicated by the darkening of the precipitate. Chemical analyses were carried out at the same periods as in the first test series.

Specimens for analysis were obtained by forcing hydrogen into the vessel through one tube and taking the water thus being squeezed out from the second tube. In this way air was prevented from getting into the experimental vessel. Results of the chemical analysis of mixtures of sea and alkaline layer waters are presented in Table 2.

As will be seen in the table, there was no sulfate reduction in the control. In mixtures consisting of 30% sea and 70% layer water the sulfate content after 26 days decreased from 928 to 576 mg/l and after 41 days to 66 mg/l. After 94 days the content increased slightly. This was probably due to air getting in when a specimen was taken for chemical analysis.

Table 1. Results of the Chemical Analyses of Hydrogen Sulfate Reduction

Mixture	Duration in days	Sulfates mg/l	Hydrogen sulfide
30% + 70% layer water	Commencement of test	928	
	26	576	119
	41	66	296
	94	176	249
	109	190	241
50% + 50% layer water	Commencement of test	1523	—
	26	700	277
	41	397	399
	94	42	546
	109	40	518

Table 2. Results of Chemical Analyses of Hydrogen Sulfate Reduction

Mixture	Duration in days	Sulfate mg/l	Hydrogen sulfide
20% sea + 80% fresh water	Commencement of test	534	—
	168	312	141
	237	252	112
	338	196	158
50% sea + 50% fresh water	Commencement of test	1336	—
	168	1136	101
	237	1148	96
	338	980	128

The results of the chemical analysis of the mixture in the ratio of 1 : 1 show that after 109 days the sulfates were almost entirely reduced. At the same time the hydrogen sulfide content in the first test variation was 241 mg/l and in the second as high as 518 mg/l. Such a picture we (Malyshek and Gasanov, 1955) only observed when sodium lactate was added to the water mixtures; when oils with different degrees of activity were added this was not observed (Malyshek and Gasanov, 1956).*

SUMMARY

1. When sulfates are introduced into layer water together with sea water they may act as a source of biogenic formation of hydrogen sulfide in the presence of molecular hydrogen.

2. Hydrogen in oil gases may act as a source of energy for sulfate-reducing bacteria.

3. In electrolytic corrosion of oil equipment in the layer water hydrogen is released in the cathode space and may thus act as a source of hydrogen reduction of sulfates to hydrogen sulfide in the pipe and trans-pipe space as a result of the activity of desulfurating organisms.

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THE CAUSE OF REDDENING OF SALT WATER IN SOME SALT LAKES *

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From the literature data and our own investigations it is now practically certain that the same cause is at the root of three phenomena: the reddening of salt water of salt lakes, the reddening of highly salted products (fish, shot birds) and salted industrial raw materials (leather, hide, gut).

Our investigations carried out with Emel'yanchik (Kurochkin and Emel'yanchik, 1935, 1936, 1937a, 1937b) confirmed the discovery of Harrison and Kennedy (1922) that the specific causative agent of the reddening of the above-mentioned salted materials in Serratia salinaria. Kurochkin (1938) demonstrated that this organism occurs in the lake salts of Europe, Asia, and Africa and with the salt is deposited on materials that are to be salted. The question of the reddening of salt water has not yet been sufficiently investigated and is in fact debatable (Isachenko, 1951; Rudenko, 1957); moreover, not a single author has suggested S. salinaria as a specific causative agent of this process.

We began the investigation by studying the salt water of Lake Tinaki. During 1957-1958 specimens were regularly taken from the same four places: the center of the lake, from two coastal zones and from a regeneration basin separated from the lake by a waterproof wall. In the basin used sludge was kept for 4-5 years. The sludge was covered with salt water. This water had a constant high salinity and a density of 29°-30° Bohm (Bogomolov, 1958) and in summer underwent intensive reddening. The salt water in the lake itself on the other hand, in recent years had been periodically freshened (Rudenko, 1957); its density fluctuated between 12° and 24° Bohm. During the period of investigation it reddened only in July 1957 and 1958.

PROCEDURE

Preparations were made from salt water as soon as it was delivered to the laboratory. Dunaliella salina and Astremonas gracilis appeared in threes and fours but more often singly or not at all even in salt water of a bright pink color. This shows that they were not the cause of the reddening of salt water. This conclusion contradicts that of Linevich (1929).

In the bacteriological investigations we took into account the ecological physiology of the microflora. To determine the presence of halophiles sowings were made on a Hottinger broth with 25% sodium chloride (salt broth). Before sterilization, a glass rod wrapped in filter paper was inserted into each tube. Part of the wrapped rod remained above the broth level (Harrison and Kennedy, 1922). Salt water was introduced in ten decreasing doses (method of marginal dilutions). At the same time, 1.0-0.1-0.01-0.001 ml and in the summer period even up to 0.00001 ml of salt water was imposed on the surface of an agar salt broth pH 7.2. This salt agar was poured in thick layers onto plates wrapped in paper. Cultivation was carried out at 37°. Visible signs of growth of red halophiles in the salt broth appeared with great constancy. It may here be remarked that the amount of material sown influences the rate at which the observable signs of growth appear; this agrees with the literature data (Rudzit, 1951).

Approximately 5-6 weeks after sowing, the tubes, when placed in a row, had a similar, very characteristic, appearance. A sign of the development of halophiles was the red zone on the paper above the broth level. The tubes with developed halophiles differed markedly from those where development did not occur. For the titer of red halophiles, the smallest quantity of salt water was taken which in the given conditions produced a reddening of the paper. The broth became slightly turbid but did not turn red. Along the side of the tube above the broth level a bacterial ring was discernable which gradually turned pink. At the same time a red bacterial precipitate appeared at the bottom of the tube.

It is inadvisable to lower the salt concentration in the broth to 20% as then, apart from the red halophiles, nonpigmented organisms multiply and reduce the chromogenic function of the halophiles. We made such an observation previoulsy (Kurochkin and Emel'yanchik, 1937b).

In order to discover which red halophiles caused the paper to redden and form a red precipitate, we studied smears and made sowings on salt agar.

Smears were prepared in a drop of water with 25% NaCl to prevent plasmolysis of S. salinaria. After drying in air and fixing over a flame the smears were stained with methyl violet-acetic acid (Kurochkin and Emel'yanchik, 1935).

This method of staining once is simpler than the twofold staining of Harrison and Kennedy (1922), Dmitrievskaya (1934), and Lochhead (1934). Admittedly, the preparation contains salt crystals, but they contribute to the study of the exceptional individuality of the morphology of S. salinaria. Cells of this microorganism accumulate in large numbers along the edge of the salt crystal and assume odd shapes: triangular, crescent shapes and so on. The Penso method (1955) proposed for the staining of halophiles is unsuitable

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as S. salinaria turns into an amorphous precipitate stained red by fuchsin.

In smears from the red zone of the paper and from the red precipitate, the same polymorphism of bacterial cells was observed as that which Harrison and Kennedy described of S. salinaria. These characteristics have been incorporated into the Berdzhe (1936) and Krasil'nikov (1949) determinators. In our material three forms of this organism predominated: spherical (nearly 2 μ in diameter) and ovoid ($2\times3\mu$). The rod-shaped ($3-4\times1\mu$) were less frequently encountered. All these forms could be stained a lilac but not a violet color.

Subcultures from the red zone of the paper, from a slightly turbid broth or from the red precipitate when inoculated on salt agar, after several days, produced only typical colonies of S. salinaria. This shows that the reddening of the paper in this medium is typical and specific. The medium is a differential-diagnostic and enables the salt water titer to be determined in relation to Serratia salinaria. The ability of this organism to propagate in exceedingly salty media and to produce a red pigment makes its identification much simpler.

The high salinity of the agar (25%) makes it bacteriostatic for a number of moderate halophiles, in particular for the nonpigmented halophile vibrion, colonies of which develop on subculturing loops from the same tubes with salt broth on agar with 10% salt. This medium in its turn has a bacteriostatic effect on the predominating majority of S. salinaria strains. Thus, S. salinaria belong to Halobacteria, i.e., microorganisms which require an extremely salty medium (Horowitz-Vlasova, 1931).

Every time the salt broth was inoculated, salt water was added to the surface of the salt agar in the plate. As red halophiles develop better on the surface of dense media (Flanneri, 1956) the index was thus determined by the number of S. salinaria cells in 1 ml of salt water.

At 37° a barely noticeable growth appeared on the fifth, tenth, or fifteenth day depending on the amount of material sown and on the individual peculiarities of the S. salinaria strain. The colonies gradually turned red (raspberry hue) and occasionally cherry red. They retained the color for indefinite periods. The agar remained uncolored. The colonies were in the shape of regular discs with a slightly convex surface. The diameter of the colonies was in the range of 0.5–0.3 mm. They all consisted of S. salinaria and remained viable under these conditions for several months. Generally there were no colonies of other microorganisms.

It should be remarked that a strain of this organism isolated from sowings of salt from Lake Tavolzhan (Altai region) formed colonies which were a milky-pink color. These strains in a milk-salt medium brought about a lightening of casein which is characteristic of Ps. cutirubra (Venkataraman and Sreenivasan, 1956). A small number of milky-pink colonies were also obtained from sowings of salt water from the regeneration basin of Lake Tinaki, and from salt water of lakes El'ton and Burlinsk (Altai). In the latter three, raspberry-colored colonies of S. salinaria predominated.

The isolated strains of S. salinaria, including those forming milky-pink colonies, reproduced an experimental "fuchsin" on pasteurized slices of salted perch.

It was found that the number of S. salinaria increased as the salt water in the regeneration basin (Table 1) and in lake Tinaki turned red.

This correspondence between the sharp increase in the number of S. salinaria and the reddening of salt water demonstrates the etiological role of this organism in the process. In November, when the salt water in the regeneration basin still retained a pink color, the number of S. salinaria decreased. Apparently the color of salt water at this period is a residual phenomenon and is probably caused by dead S. salinaria cells and a specific pigment.

Reddening of salt water reproduced in laboratory conditions was found in every case to be due to S. salinaria. Tests were carried out in two variations.

I. Colorless salt water taken from the center of the lake in May 1957 was poured into three retorts, 500 ml into each. A layer of Tinak salt—2 cm thick—was placed at the bottom of each retort. Glass rods wrapped in filter paper were inserted into the retorts and the lot were sterilized in an autoclave at 120° for 30 minutes. One retort served as control for sterility. Over a period of two years (duration of observation) no changes occurred in it.

Into the other retort a pure strain of S. salinaria isolated from salt water of Lake Tinaki was introduced. To the third retort 5 ml of pink salt water taken from the regeneration basin was added. The retorts

Table 1. Titer and Index of Serratia salinaria in Salt Water of the Regeneration Basin and in Salt Water Taken from the Center of Lake Tinaki during 1958

Month	From the regeneration basin			From the lake		
	coloration of salt water	titer	index	coloration of salt water	titer	index
January	Colorless	0.1	50	Colorless	0.1	60
February	"	0.01	850	"	0.1	30
April	"	0.001	5 800	"	1.0	20
May	"	0.001	7 000	"	0.1	400
June	Pink	0.00001	110 000	"	0.001	18 000
July	Bright pink	0.000001	1600 000	Pinkish hue	0.00001	102 400
August	Pink	0.00001	300 000	Colorless	0.001	7 600
September	"	0.001	20 000	"	0.1	100
November	Pale pink	0.01	750	"	0.01	780
December	Colorless	0.01	700	"	0.1	110

were kept at room temperature. The nutrient medium reproduced to a considerable extent the conditions obtaining in the lake.

After two months, the paper over the salt water in the test retorts acquired a pink color. After six months, the paper turned red and the salt water, a pale pink. At the bottom of the retorts under the layer of salt a small red precipitate appeared. Sowings from the second and third retorts on a salt agar produced only typical colonies of S. salinaria. Subcultures from the third retort on agar with 10% salt did not result in the development of S. salinaria colonies. Instead colonies of unpigmented halophile vibrion developed. Sowings on this medium from the second retort did not develop.

This demonstrates that Tinak salt water, in conditions imitating the ecological peculiarities of the lake, acquires a pink color as a result of the multiplication of S. salinaria.

II. In the second test variation, reddening of salt water was experimentally reproduced in the following way: 8 ml of colorless salt water taken from Lake Tinaki in January 1958 was added to plates with lactic-salt agar, with 20 and 25% salt, and to plates with salt agar. In another test carried out under the same conditions, 8 ml of sterilized salt water and one loop of a pure S. salinaria strain were added to the plates. The plates were kept at 37° in a semiopen exsiccator which had salt water at the bottom (Petrova, 1936).

The salt water turned red after 3–4 weeks. Smears obtained from plates to which sterilized salt water and a pure S. salinaria strain had been added, after staining with methyl violet-acetic acid, revealed S. salinaria cells. Subcultures on a salt agar produced colonies only of S. salinaria. Sowings on agar with 10% salt showed no development.

In the test in which crude salt water had been used, an abundance of S. salinaria cells and a small number of vibrions and violet-colored cocci could be seen through a microscope in the red bacterial deposit. Sowings on salt agar produced an abundant growth of S. salinaria colonies and a very small number of red colonies of halophile cocci (Petrova, 1929). Sowings on agar with 10% salt caused unpigmented halophile vibrion and a few unstained colonies or brownish colonies of halophile and halotolerant rods to develop. Thus, even the second test variation demonstrated that the reddening of salt water in laboratory conditions is brought about by the abundant propagation of S. salinaria.

In order to increase the number of specimens studied, we also investigated, by the same methods, the salt water and salt of lakes El'ton and Baskunchak. We already know from the literature that the brine of a number of salt lakes in Altai undergo a reddening (Isachenko, 1951, Vetlina, 1958). Specimens of salt water and salt from some of the Altai lakes were sent at our request by the senior scientific worker of the West-Siberian Branch of the Academy of Sciences of the USSR Yu. P. Nikol'skaya and V. G. Edgarhead of the Central Laboratory of the Mikhailov Soda Combine, to both of whom we are deeply grateful.

Only the salt water of Lake Burlinsk (taken on 5 August 1958) was of a pink color. The other specimens were colorless as they had been taken at the end of summer or in the autumn when the red color of the salt water either faded or disappeared altogether. The investigation was carried out by the method of marginal dilutions and by pouring the specimens on the same media. The results are presented in Table 2.

From Table 2 it will be seen that the number of S. salinaria varies; it is particularly high in the pink salt water of Lake Burlinskii—320,000 in 1 ml and a titer of 0.00001 ml. Sowings of reddened paper on salt agar gave rise to Serratia salinaria colonies. Sowings from the same tubes on agar with 10% salt, as a rule, produced colonies of halophile unpigmented vibrion.

These salt water specimens were subsequently reddened in laboratory conditions.

An 8-ml specimen of each salt water was poured onto the surface of salt agar in plates which were then placed in an exsiccator at the bottom of which was salt water. After 3–5 weeks at 37° the salt water turned red. A microscopic examination of smears and sowings on salt agar revealed an abundant growth of S. salinaria.

Under these conditions, only the salt water of Lake Yarovoe did not turn red, nor were any S. salinaria found in it although it did contain the halophile unpigmented vibrion. Isachenko (1951) demonstrated that in the coastal area of this lake, red stains consist of purple organisms. The role these organisms play in the reddening of salt water of certain lakes was pointed out by Rubenchik and Goikherman (1939) and Jannasch (1959). If in the reddening of salt water other organisms play an etiological role this is probably due to the combination of various salts and their different concentrations in salt water.

Table 2. Titer and Index of Serratia salinaria in Salt Waters of Some Lakes

Date on which specimen was taken	Lake	Titer	Index
24 Sept. 1958	El'ton	0.1	90
30 Jan. 1959	Baskunchak	0.1	100
5 July 1958	Burlinskii	0.00001	320,000
13 Oct. 1958	Malinovoe, southern stretch	5.0	—
13 Oct. 1958	Malinovoe, northern stretch	0.1	95
13 Oct. 1958	Malinovoe, section 1	0.1	80
13 Oct. 1958	Pechatnoe	1.0	—
10 Oct. 1958	Yarovoe	—	—

The following peculiarity should also be mentioned. Strains of S. salinaria isolated from the salt water of Lake Pechatnoe (Altai) developed on agar with 10–15% salt but their growth was noticeably inhibited on agar with 20–25% salt. This shows that different strains of this organism tolerate osmotic pressures of different magnitudes.

SUMMARY

1. A correlation has been established between the seasonal increase in Serratia salinaria of salt water and the seasonal reddening of salt waters of Lake Tinaki (Astrakhan region) and Lake Burlinskii (Altai).

2. Sterilized salt water of Lake Tinaki becomes red when a pure strain of S. salinaria is introduced, owing to the vigorous reproduction of this organism.

3. Salt water of Lakes Tinaki, El'ton, Baskunchak, Malinovoe, and Pechatnoe, when imposed on the surface of sterile agar (25% salt), turn red after a certain lapse of time. This reddening is caused by S. salinaria.

4. The above results demonstrate the etiological role of S. salinaria in the reddening of salt waters of the investigated lakes.

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OLIGODYNAMIC ACTION OF CHEMICALLY PURE METALLIC SILVER

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It is known that silver has a bactericidal action. This property of the metal is widely exploited in practice (Kul'skii, 1955; Boitsov, 1946). The greater the surface contact of silver with the medium, the more effective the bactericidal action. That is why silver is generally used in a severely dispersed condition (for example, Collargol). The oligodynamic action of silver depends not only on the surface area but also on its physicochemical condition.

The literature (Ermolaev, 1935; Plevako, 1935; Lazarev, 1935) describes the effect of surface films of Ag_2O, AgCl, AgBr, and AgI on the bactericidal action of silver. However, chemically pure metallic silver, when kept in the usual conditions, does not form these films but readily combines with the hydrogen sulfide in the air to form sulfide films (Boitsov, 1936; Karyakin and Angelov, 1955; Slavinskii, 1952). Yet in spite of the fact that the sulfide film is particularly characteristic of silver, its effect on the bactericidal action of silver has not been investigated.

It is quite natural to assume that the oligodynamic action of silver depends also on physicochemical properties of the metal itself. It is known for instance that during plastic deformation its physical condition undergoes a change (Kuznetsov, 1941). The crystal lattice of the metal becomes deformed, energy is absorbed and as a result its properties change. The chemical activity of deformed metal is slightly greater than that of undeformed. With an increase in chemical activity there ought to be an increase in the yield of silver ions in the solution and this may intensity the bactericidal action.

The present work describes an attempt to elucidate the effect of: a sulfide film, plastic deformation and of the application of an electric field on the bactericidal action of silver.

METHODS

The preparation of colloids of a definite particle size and surface condition is extremely difficult. Hence in this investigation we used discs of chemically pure silver $20 \times 1.8 \times 0.8$ mm in size. The chemically pure silver was obtained from commercial "table silver" by Kerlyo (Karyakin and Angelov, 1955). Unlike the compounds Ag_2O, AgCl, AgBr, and AgI which are readily soluble in ammonia (Karyakin and Angelov, 1955; Ermolaev and Rotmistrova, 1935) the sulfide film does not dissolve in it. Therefore before the tests the surface of the discs was cleaned with 35% nitric acid for three minutes at 50°, after which the plates were washed and then immersed in a 72% ethyl alcohol to preserve sterility.

The antibacterial properties of silver were tested on Eschericha coli. 0.5 ml of a suspension from a 24-hr E. coli culture containing 50,000 cells was placed in a Petri dish and 20 ml of molten MPA was added. After the agar had set, the sterile silver discs were placed on the plate. The surface contact of the plates with MPA measured by a universal measuring microscope UIM-21 was a constant 0.5 cm^2.

The bactericidal action of silver was determined by the width of the zone of complete inhibition of development of E. coli around the silver discs. The width of the zone was measured by the UIM-21 from every side of the disc and then the average values were taken.

RESULTS

It was found that the bactericidal properties of silver depend, in the main, on the condition of the surface of the discs. The purer the surface of the silver the more pronounced its antibacterial properties.

To elucidate the effect of surface films, tests were carried out in which the discs were covered with a sulfide film.

A dark brown film, deposited on the discs by placing them in a hydrogen sulfide saturated water solution, was obliterated by nitric acid to varying depths or completely removed. It was found that on increasing the thickness of the film the bactericidal properties of silver severely diminished. As an example test No. 11 is presented in Fig. 1.

Figure 1 shows that one sulfide film caused a two-fold reduction in the bactericidal properties and that after exposure to acid for 5–6 minutes the sulfide film was completely eliminated and the width of the zone ceased to increase.

An exactly similar effect is produced by a film formed by natural means in air.

To elucidate the effect of the condition of the metal itself on the antibacterial properties, the bactericidal actions of plastically deformed (by rolling) and annealed silver were compared. Samples were prepared by rolling to different degrees of deformation. Half the samples were annealed in a vacuum at 400° for 30 minutes to eliminate the deformation and were then subjected to the usual chemical cleaning together with the deformed samples.

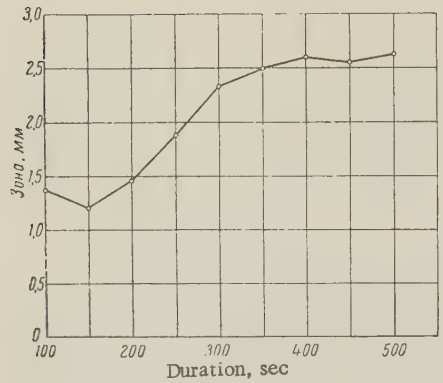

Fig. 1. Effect of sulfide film on the oligodynamic properties of silver.

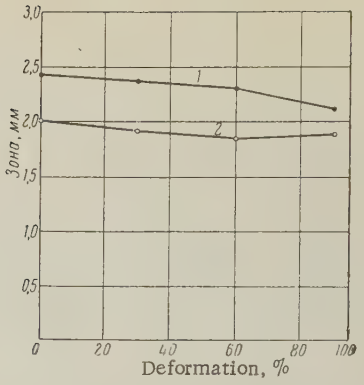

Fig. 2. Effect of plastic deformation on the oligodynamic properties of silver. (1) Deformed samples (2) annealed samples.

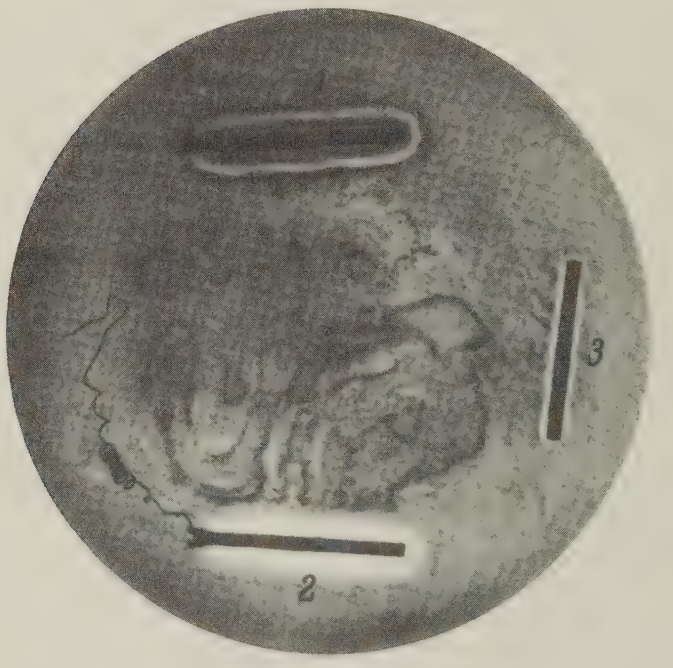

Fig. 3. Arrangement of discs in a Petri plate. Actual size.

Figure 2 shows that when the deformation is nil, the width of the zone is variable. This can only be explained by the different treatment of the samples. It will be observed that as the deformation is increased this difference somewhat diminishes.

To elucidate the effect of the discharge of silver into the medium, tests were carried oût in which the discs under investigation were given different potentials. The potential was provided by a pressure divider to a closed Petri plate and thence directly to the thermostat. A third disc placed in the plate but outside the active zone of the electrical field served as control. As might have been expected, under these conditions, the yield of silver increased sharply; moreover, it rose as the applied pressure was increased.

At high potentials the output of silver was so considerable that around the positive disc the agar became covered with a thin film of silver. It was found that irrespective of the magnitude of the applied potential, the zone on the positive disc was approximately twice that on the control. The small negative potential reduced the zone; at potentials over 0.5 v the zone on the negative disc was greater than on the control and increased in proportion to increases in potential.

Figure 3 presents a photograph of the discs with the zones in actual size and Figs. 4, 5, and 6, magnified 25 times.

Figure 3 shows the general appearance of one of the tests in which there were different potentials between the discs under investigation. The difference in poten-

Fig. 5. Zone of suppression and type of growth around the positive disc. Mag. 25x.

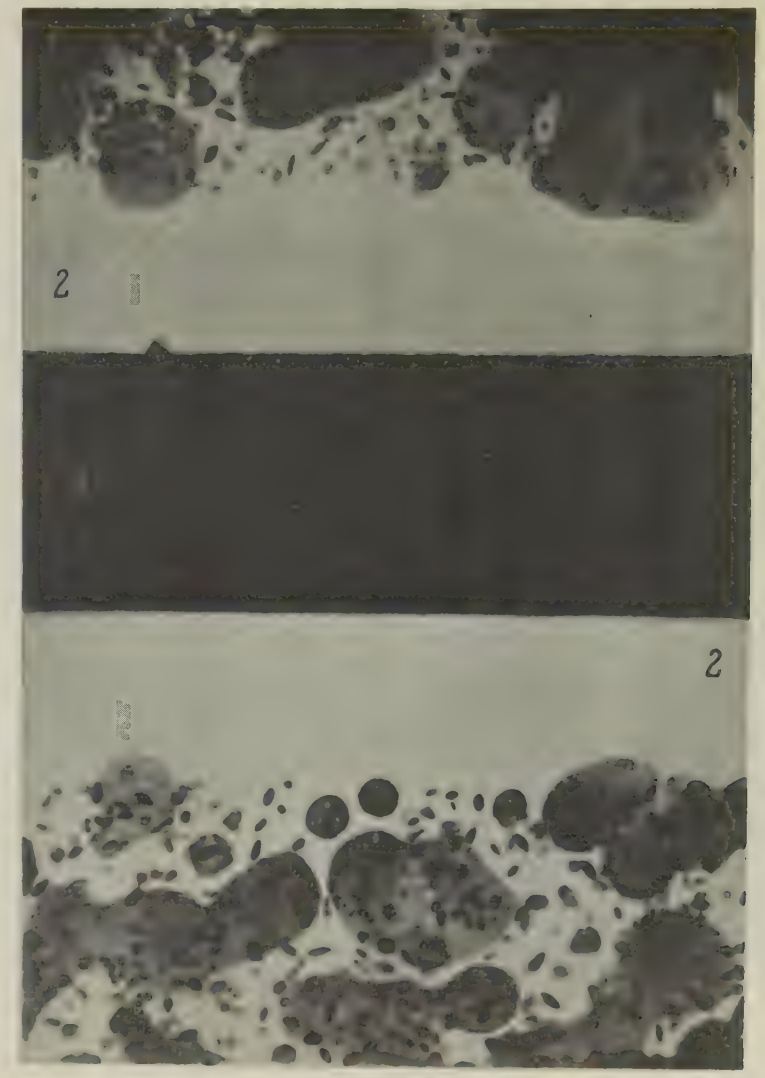

Fig. 4. Zone of suppression and type of growth around the positive disc. Mag. 25x.

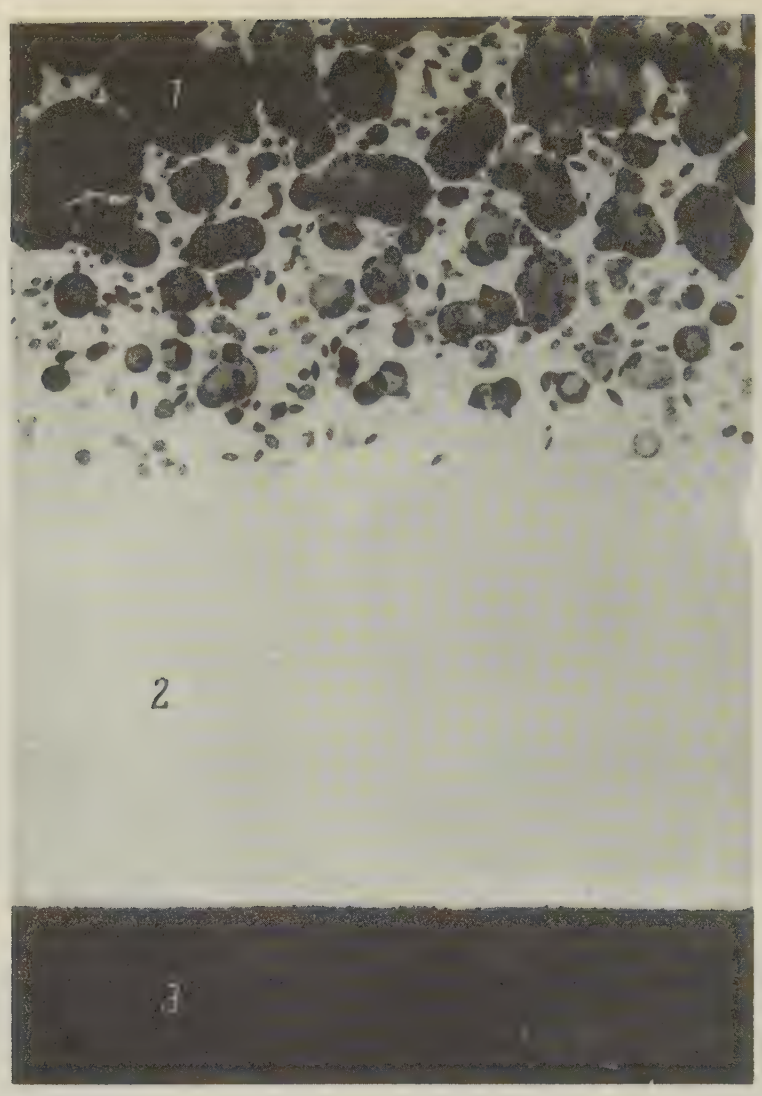

Fig. 6. Zone of suppression and type of growth around the negative disc. Mag. 25×.

tial was 1.2 v. Pressure was applied to the ends of the discs which were bent back along insulated wires which can be clearly seen in the diagram. A positive potential was applied to disc 1, a negative to disc 2 and the third disc served as control. Around the positive disc a silver film can be clearly seen on the surface of the agar. Around both the negative and positive discs the zones of suppression are considerably wider than around the control.

Figure 4 shows the zone of growth suppression around the control disc 3. Here, the zone of complete suppression (2) quite abruptly passes into the zone of normal bacterial development.

Figure 5 shows the zone around the positive disc 3. Here the zone of complete suppression (2) is very suddenly replaced by the zone of active growth (1). And finally, around the negative disc (Figs. 6, 3) the zone of complete suppression (2) (which was used for measuring the size of the zone) is replaced by the zone of partial suppression which gradually merges into the zone of normal growth (1).

DISCUSSION

The data regarding the effect of sulfide films on the bactericidal properties of silver shows that when using silver articles it is necessary to bear in mind the formation of a Ag_2S film which reduced the bactericidal action of the metal. It is further recommended that silver preparations be kept in a hermetically sealed dish because if left in air they react with H_2S and moisture to produce silver sulfide which in turn reduces the effectiveness of silver preparations.

The immutability of the bactericidal properties when the physical properties of the metal change (tests of plastic deformation) convincingly demonstrate that it is not the metal itself (disc) which has the bactericidal action but the ions which come out in solution. This conforms with the ion theory of the bactericidal action

f silver. It is also supported by results of tests with ifferential potentials between the discs.

SUMMARY

1. Sulfide film considerably reduces the bactericidal ction of silver.
2. Plastic deformation has no substantial effect on ıe bactericidal properties of silver.
3. Of the investigated factors the electrical field has ıe greatest effect on the antibacterial properties of ilver.

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COMPARATIVE STUDY OF ACTINOMYCES RIMOSUS (OXYTETRACYCLINE PRODUCER) AND EXPERIMENTALLY INDUCED VARIANTS

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The most promising group of microorganisms for antibiotic production are the actinomycetes. At the same time their great tendency for variation is well known, and hence it is of particular importance to determine exactly what characteristics and properties of the organism are the most stable.

Inadequate study of the extent of variability in antibiotic-producing actinomycetes frequently results in describing and establishing forms freshly isolated from nature as new species; this confuses the classification of the actinomycetes and hinders work on searching for new antibiotics.

The most effective method for constructing a system of natural classification is that of experimental variability, as was demonstrated in studies contained in the monograph of N. A. Krasil'nikov (1958).

The present paper deals with variability in Actinomyces rimosus. Four strains, obtained from culture collections in various countries, were studied.

In order to determine the type and extent of variability under laboratory conditions in the oxytetracycline producer, we considered such factors as temperature, substrate on which cultures were grown and stored, frequency of transfer, method of growth—on solid media or in submerged culture on liquid media, etc. The following special factors were employed in determining A. rimosus variability: cultivation of the original strains at the sublethal temperatures –37, 40–42, and 45° on various solid media; cultivation on solid media containing chlortetracycline (30, 100, 450 units/ml); cultivation on solid media with initial pH 5.8–8.5; action on cultures of ultrasound.

Morphological and cultural properties of the strains were studied on natural media: potato, corn, pea, meat-peptone, and on synthetic media: glucose-asparagine, modified Czapek (CP-1) and CP-10. The composition is given below of the agarized media (in g/liter) employed most frequently in the study.

1. Potato agar: 200 g of potatoes washed clean are put through a fine grinder. The material obtained is thrice washed with warm tap water and filtered through a double layer of gauze. The potato pulp is discarded, the wash waters are allowed to stand so that the starch can settle, then decanted and the water brought to a liter; the following are added: $(NH_4)_2SO_4$—3.5; NaCl—3.0; chalk—2.0; glucose—10.0; when stirring the mixture the settled starch is added.

2. Corn agar: corn extract—20.0; $(NH_4)_2SO_4$—2.0; NaCl—5.0; glucose—5.0; chalk—2.0; starch—10.

3. Synthetic medium CP-1: $MgSO_4$—0.5; K_2HPO_4—0.5; NaCl—0.5; KNO_3—1.0; glucose—20.0.

To the media was added 20 g agar-agar.

The study of variability was conducted chiefly on the potato medium, which was the best of all the media we tested for revealing the variability of A. rimosus. The special features of each variant, beyond the relationship to the previous cultural conditions, appear most characteristically on this medium. The synthetic media were found to be unsuitable for demonstrating the variability of the oxytetracycline producer.

It is highly significant that various factors to which A. rimosus was subjected caused the formation of analogous variants. Definite types of variants that appeared under conditions of laboratory culture also appeared when such factors as sublethal temperature, ultrasound, chlortetracycline, etc., were employed (Fig. 1). A. rimosus variants described in the liter-

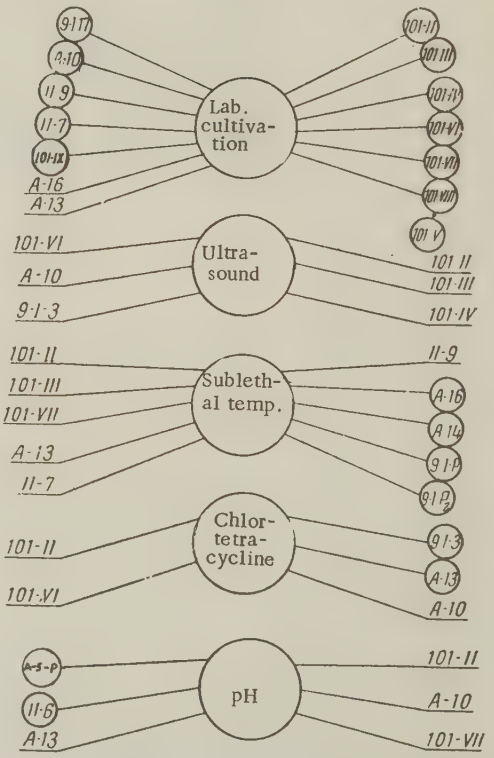

Fig. 1. Diagram of variant formation in different Actinomyces rimosus strains under the influence of various factors (Original strains 101, A, 9-1, II). In the small circles are indicated the variants appearing for the first time under the influence of the given factor.

ature (Teillon, 1953; Borenstain and Wolf, 1956; Mindlin and Alikhanyan, 1958), obtained by ultraviolet irradiation, resemble in a number of characteristics the forms appearing under our conditions.

The original A. rimosus strains had spiral sporophores with 3–6 turns; shape of spores was oval and elongated. Aerial mycelium on most media was whitish. Several differences in cultural characteristics were noted between strains: in the activity of aerial mycelium formation, in color of substrate mycelium (from sandy beige to yellowish beige), in the characteristic of fissuring or "exfoliation" of mycelium typical of A. rimosus.

The variants obtained from the original strains differ from the latter and among themselves in a number of morphological and cultural characteristics, on the basis of which we subdivided them into five basic types (Table 1).

The first type comprised variants (A-14, 101-II, A-13, 101-IV, 101-VII) in which aerial mycelium was lacking on the media we used, substrate mycelium light sand color to dark brown, soluble pigment light brown to yellowish brown.

The second type comprised variants (101-III, 101-V, A-10, 9-1-3, II-7) with yellowish-grayish serial mycelium, substrate mycelium dark brown, soluble pigment light brown to dark brown. The characteristic of fissuring of mycelium was exhibited in varying degree.

The third type included variant 9-1-R, forming flesh-pink aerial mycelium and orange-red substrate mycelium on a number of media. The media become brownish.

The fourth type included variant 101-VI, for which very small colonies were typical, as were absence of growth or extremely poor growth on a number of synthetic media. No aerial mycelium was formed on any of the media used, substrate mycelium black, soluble pigment dark umber.

The fifth type comprises variants (101-VIII, 101-IX, A-5-II) of the proactinomycete type. They are distinguished by dough-like consistency of colonies and early fragmentation of mycelium into rod-shaped and coccus-shaped elements. Aerial mycelium was absent on all media, substrate mycelium colorless and light cream in variants 101-IX and A-5-II and dark chestnut in variant 101-VIII, the latter coloring most organic media dark brown.

It is of interest that the various original A. rimosus strains we studied form morphologically similar variant types in the majority of cases.

From all the strains, variants with yellowish gray aerial mycelium and brown substrate mycelium were encountered particularly often. No less often there appeared from all the parent forms asporogenous variants with sandy to dark brown substrate mycelium. Variants of the proactinomycete type and variants for which small colonies and black substrate mycelium were typical appeared more rarely. The appearance of unpigmented variants was a rare phenomenon. In only one original strain did a form appear (9-1-R) which was not observed in the other parent cultures.

We consider variant 9-1-R with flesh-pink aerial mycelium and orange-red substrate mycelium to be unique, since we have not encountered a description of this type of A. rimosus variant in the literature.

Certain differences of enzyme activity were noted in the cultures studied, indicated by varying ability to

Table 1. Morphological and Cultural Properties of Original and Variant Actinomyces rimosus Strains (Potato agar)

Culture	Sporophores and spores	Colony diam., mm*	Aerial mycelium**	Substrate mycelium	Soluble pigment	Remarks
Original strains 101, A, 9-1, II	Spiral; oval and elongated	16	Poorly, moderately, and well developed; like flour, whitish	Beige to yellowish-orange	Dark chestnut (101-VIII)	Fissuring of mycelium typical of Actinomyces rimosus especially marked in strains 9-1 and II
Type I—variants 101-II, A-13, 101-IV, 101-VII	None	13–15	Lacking	Sandy to dark brown	Light brown to yellowish-brown	
Type II—variants 101-III, 101-V, A-10; 9-1-3; 11-7	Spiral; oval, elongated, and rod-shaped (in var. 101-V)	13–15	Moderately developed, velvety, yellowish-gray	Dark brown	Light brown to dark brown	Fissuring of mycelium strongly marked in variants 9-1-3 and II-7
Type III—variant 9-1-R	Spiral; oval and elongated	14, 5	Well developed, velvety, flesh-pink	Orange-red	Light brown and umber	
Type IV—variant 101-VI	None	7	Lacking	Black	Brownish	
Type V—variants 101-VIII, 101-IX, A-5-II	None	9–13	Lacking	Colorless (101-IX), cream (A-5-II)	Dark umber	Variants of proactinomycete type, characterized by dough-like consistency of colonies and rapid fragmentation of mycelium into rod-shaped and coccus-shaped elements

*Average size of 5 colonies (stab culture) 10 days old
**Color of aerial and substrate mycelium and soluble pigment was determined from the color scale of A. S. Bondartsev (1954)

liquefy gelatin, coagulate or peptonize milk, hydrolyze starch, reduce nitrate to nitrite, etc. However, these differences were more quantitative in nature than qualitative. Variant 101-VI is distinguished by an almost complete lack of the enzyme activity indicated.

All the cultures, with a few exceptions, behave almost alike in the ability to assimilate various carbon sources (12 carbon-containing compounds were employed). Variant 101-VII is different in that it does not grow at all on medium containing lactose and raffinose, and variant 101-VIII utilizes these substances very poorly.

Among the physiological properties, oxytetracycline production by the cultures merits special attention. The cultures were grown on different media —corn and synthetic media—in flasks on a shaker.

It follows from Fig. 2 that most of the variants (17 of 20) were more active than the original cultures. The activity of some variants exceeded that of the parent culture 13 times (80 units/ml in original culture and 1080 units/ml in variant II-7). The medium exerted a great effect on antibiotic formation by a given strain. Thus, variant A-14 produced 60 units/ml on corn medium containing sodium nitrate as nitrogen source, and 600 units/ml on medium of the same composition but where nitrate nitrogen was replaced by ammonium nitrogen.

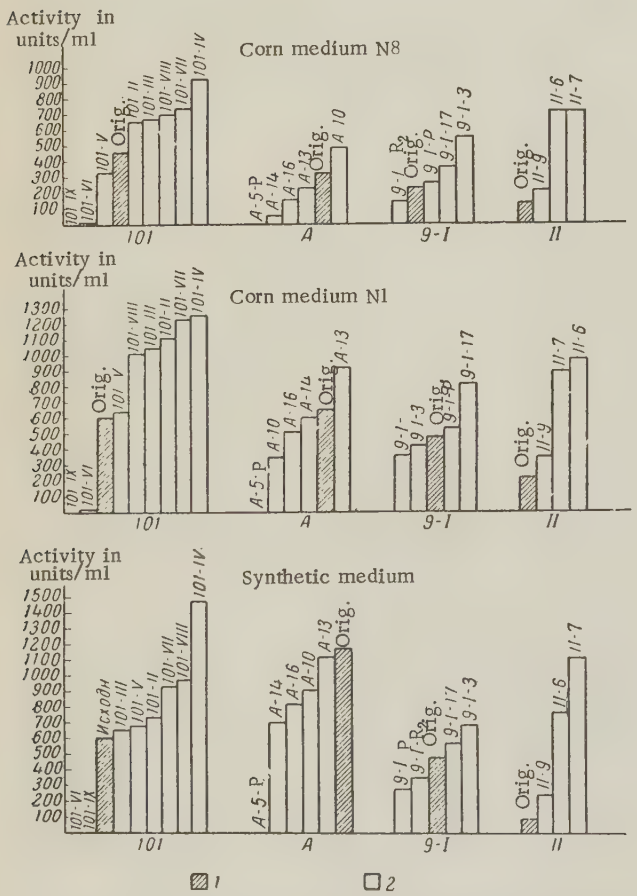

Fig. 2. Relative activity of original and variant Actinomyces rimosus strains on various media. 1) Original strain; 2) variants.

Three variants of the number we studied formed traces of oxytetracycline (5–20 units/ml) on all media employed. Of these, variant 101-VI is evidently a physiologically deficient form. It grows poorly and forms no antibiotic on a number of synthetic and natural media (Rudaya, 1958). Variants 101-IX and A-5-II of the proactinomycete type are virtually inactive.

The antibiotic production process on the media employed is characterized by certain indexes that are somewhat different in the various cultures. These indexes include type and intensity of growth, pH, frothing, and pigmentation of the culture fluid. The latter is as a rule correlated with the activity—the more intense the pigmentation of the culture fluid, the greater the activity.

Determination of the antibacterial spectrum* (a number of saprophytic and pathogenic bacteria) did not reveal any qualitative differences in the A. rimosus cultures. The latter inhibited growth of the test-bacteria to different degrees (Table 2).

It is known from the literature that A. rimosus, besides oxytetracycline, produces the antibiotic rimocidin, which has antifungal properties. We therefore tested our cultures on a number of saprophytic and pathogenic fungi. Here it was found that variants 101-VI, 101-IX, and A-5-II, which are inactive toward bacteria, inhibit growth of fungi just as actively as the variants active towards bacteria. Hence it may be assumed that variants 101-VI, 101-IX, and A-5-II, having lost the ability to produce oxytetracycline in the process of variation, retained the capacity to produce rimocidin.

Our assumption was confirmed by chromatogram and spectrogram data.

We employed ascending paper chromatography in a solvent system consisting of 2 parts butyl alcohol, 1 part distilled water, and 1 part glacial acetic acid, and descending chromatography in a water-saturated ethyl acetate system. Culture fluid separated from mycelium was applied to the paper. A mixture of standard solutions of oxytetracycline, tetracycline, and chlortetracycline was applied as control.

Chromatograms were developed under ultraviolet light (radiation source was a PRK-4 lamp with UFS-3 filter), in which oxytetracycline shows a characteristic bright yellow color (Regna and Solomons, 1950; Berti and Cima, 1955). Results of the tests showed that all the cultures, with the exception of certain variants, produce oxytetracycline: spots obtained on chromatographing were at the level of the oxytetracycline spot. No oxytetracycline was found in 101-VI, 101-IX, and A-5-II culture fluid. The spectrogram with three characteristic maxima in ultraviolet light (data of V. V. Korchagin) confirms the assumption of the formation by these variants of an antibiotic of the rimocidin type.

*Spectrum determination was carried out in the VNIIA Control Laboratory by I. S. Buyanovskaya.

Table 2. Antibacterial Spectrum of Original and Variant Actinomyces rimosus Strains (Method of serial dilutions $\times 10^{-1}$)

Test-bacteria	Orig. strain 101	Variations of strain 101	Orig. strain A	Variations of strain 101	Orig. strain 9-1	Variations of strain 9-1	Orig. strain II	Variations of strain II
Bact. typhi abd.	1600	<10—1600	800	<10—400	400	100—400	200	100—800
" paratyphi B	1600	<10—1600	800	<10—800	400	100—800	400	200—1600
" dysenter. H-Flex.	3200	<10—3200	800	<10—800	400	100—400	200	200—800
Bact. dysenter. Sonne	800	<10—800	800	<10—400	200	100—400	100	100—800
C. diphtheria	1600	<10—1600	1600	<10—800	800	200—800	400	400—1600
Bact. coli	800	<10—800	400	<10—400	200	100—200	100	100—400
Proteus vulgaris	<100	<100	<100	<100	<100	<100	<100	<100
Proteus 0 × 19	<100	<100	<100	<100	<100	<100	<100	<100
Ps. pyocyaneus	<100	<100	<100	<100	<100	<100	<100	<100
Bac. anthracoid	2000	0—4000	3200	0—3200	3200	400—3200	1600	800—3200
Bac. mycoides	4000	0—8000	2000	0—2000	1000	400—1000	800	400—800
Staph. aur. 209 P	4000	0—4000	6000	0—4000	3200	400—3200	1600	800—6000

We tested for antagonistic interaction among the cultures (Krasil'nikov, 1951) grown on a number of agar media by means of cross-inoculation.

It follows from the data obtained that neither original nor variant strains of A. rimosus had a mutually antagonistic effect and, consequently, they all reacted among themselves as strains of a single species; the antibiotic they produced did not change in nature. With regard to other species of the genus Actinomyces used as control—A. streptomycini, A. chrysomallus, A. aureofaciens—a bilateral antagonism was exhibited. All A. rimosus cultures inhibited growth of Actinomyces albus and Actinomyces aureus, themselves remaining insensitive to them.

Thus, the data on the antimicrobial spectrum and the results on antagonistic interaction in the induced variants demonstrate that, in the presence of greatly changed morphological, cultural, and physiological properties, their biological feature—of not inhibiting the growth of one another—is retained in the present case unchanged.

According to data in the literature, oxytetracycline is produced not only by A. rimosus, but, in addition, by other actinomycetes described as new species (A. griseoflavus, A. armillatus, and A. platensis).

However, on comparing the characteristics in our A. rimosus variants with the description of other oxytetracycline producers (unfortunately no original cultures were at our disposal for comparative study), a definite resemblance can be observed among them. Thus, variant II-6 and A. armillatus (Mancy-Courttillet and Pinnert-Sindico, 1954) resemble one another morphologically; they have strong wrinkled yellowish-orange substrate mycelium on organic media, colonies are surrounded by a zone of poorly developed whitish aerial mycelium, and the characteristic fissuring of mycelium typical of A. rimosus is lacking. A. platensis (MacGuire, 1954) resembles variant 9-1-17 in several characteristics. The oxytetracycline producer T-3-248 isolated by Janot, Penau, et al. (1954) resembles variant 101-IV; both are lacking in aerial mycelium, and substrate mycelium is brownish black; A. griseoflavus, according to the statement of the authors themselves (Kocho, Asker, et al., 1952), is identical to A. rimosus.

The tendency of some workers to describe the producers of already known antibiotics as new species is due to various reasons. In a number of cases it is the result of lack of knowledge of the producers' biological features, particularly their range of variability; this confuses classification.

The authors are deeply grateful to Professor N. A. Krasil'nikov for valuable advice and instructions in carrying out the present study.

SUMMARY

1. The variability of 4 Actinomyces rimosus strains has been studied. Among 20 experimentally induced variants, some resemble the original cultures closely in certain properties and some differ sharply in morphological and cultural characteristics and in antibiotic properties.

2. Identical variants can be obtained from different original A. rimosus strains, and variants of the same type can be obtained through the agency of different factors—laboratory cultivation or exposure to special agents.

3. Variants of the proactinomycete type have been isolated. This confirms the phylogenetic relationship between proactinomycetes and actinomycetes. It also indicates the great importance of the experimental variability method in constructing a system of natural classification.

4. Despite the considerable difference in morphological, cultural, and antimicrobial properties, none of the variants inhibited each other's growth or that of the original culture. This indicates that they belong to a single species, and that the "crossing" method can be used for diagnostic purposes.

5. Compounds from culture fluid of original strains and variants with antibacterial activity were shown to be identical to oxytetracycline by paper chromatography. Variants without antibacterial activity retained the capacity to produce rimocidin.

Thus, variants produced under different conditions and differing in morphological and physiological properties produce the antibiotics characteristic of the given species (A. rimosus)—oxytetracycline and rimocidin.

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ARTIFICIAL CULTURAL CONDITIONS FOR PIGMENT-COMPLEX FORMATION BY POLYPORUS

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The therapeutic preparation obtained from the wood-decomposing fungus Inonotus obliquus (Pers.) Pil., popularly termed polyporus, by treating water extracts of the fungus outgrowths contains an amorphous pigment-complex (Yakimov, et al., 1957) having a physiological action. This complex belongs to the group of high-polymer compounds of the humic acid type, but differing from the latter in its solubility in water, the absence of nitrogen, and the large number of methoxy groups (Shivrina et al., 1959).

The pigment-complex is produced by polyporus under natural conditions in the amount 15–20% of the mycelium dry weight. In water extracts of the fungus growth the pigment-complex varies from 50 to 60% of the dry residue. A characteristic property of the complex is its capacity to precipitate out from solution when the pH is changed to 2.5–3.0.

In the present paper the preliminary results are given of a study on the artificial cultural conditions for formation of the pigment-complex by the fungus.

Many fungi are known to form humic acids in culture quite readily (Sidorenko, 1957; Bejerinck, 1900; Laatsch et al., 1952; Siegel, 1954). But no one has yet studied the production of a pigment-complex identical in chemical composition to that formed by polyporus under natural conditions.

The lack of a simple method for determining the biological activity of the polyporus preparation greatly complicated the work on obtaining its active principle in artificial culture. In order to identify the pigment-complex formed in artificial culture we had to resort to the method of splitting it into simple compounds and comparing them by means of paper chromatography and column chromatography to the cleavage products of the complex formed by polyporus under natural conditions.

The technique of study, described in detail in a previous study (Lovyagina et al., 1958), was briefly as follows: Hydrochloric acid was added to the culture fluid so as to produce a concentration of 10–12% in the sample and the mixture was hydrolyzed at a pressure of 1.5 atm for 2 hours. The hydrolyzate was treated repeatedly with ethyl ether. The ether solution was first studied qualitatively by paper chromatography and then quantitatively on a column of silica gel. Five fractions were isolated, each containing compounds characteristic to it.

EXPERIMENTAL

The composition of the water-soluble, precipitable natural conditions and investigated by the technique described, is quite constant and can serve as a kind of standard in studying the precipitable pigment-complexes obtained in artificial culture (Table 1).

Table 1. Composition of Ether-Soluble Hydrolysis Products of Polyporus Pigment-Complex (in percent of dry weight of experimental sample)

Determination	Fractions from silica-gel column					Total substances eluted from column
	A acid mixt.	B pigments	C syringic acid	D vanillic acid	E p-hydroxybenzoic acid	
Minimum	25	4	15	5	1.2	50
Maximum	35	6	17	7	2.0	67
Average of 20 determinations	28	5.5	16	6	1.8	60

The polyporus culture, isolated from fungal growths, was grown in surface culture. The basic medium was a solution of the following composition (in g): KH_2PO_4 1.0; $MgSO_4$ 0.5; KCl 0.5; peptone 5.0; glucose 10 per liter tap water. The pH of the medium was ~6. The media employed to obtain the pigment-complex can be divided into two types: 1) media with addition of assumed precursors of the complex, and 2) media containing lignin in the form of birchwood sawdust.

It was found in previous studies (Lovyagina et al., 1958) that the polyporus pigment-complex is a highly condensed compound of aromatic nature, resembling in composition such compounds as the humic acids and lignin.* In the formation of the latter the pectins may also take part (Rudakov, 1949). The following compounds were employed as precursors: phenols (Gallic acid, tannin, guaiacol), aromatic hydroxy acids (syringic, vanillic, and a mixture of methoxylated derivatives of syringic and vanillic acids), methylated and methoxylated carbohydrates (rhamnose, aqueous extract of sugar beet containing pectin). Phenols and aromatic hydroxy acids were added to medium in the amount 0.1%.

The medium containing sawdust from birchwood, the principal material for pigment-complex formation under natural conditions of polyporus growth, had the

*There are indications in the literature that compounds of a polyphenol or phenol type (Kononova, 1953) may be oxidized to lignin-like or humin-like compounds.

following variants: a) medium containing sawdust from young, less woody birch twigs, b) medium containing sawdust from mature birchwood, and c) medium containing sawdust after growth upon it of brown rot fungi (Coniophora cerebella and Poria vaporaria), which decompose cellulose without affecting lignin.

The sawdust was treated with alcohol-benzene mixture to remove resins and added to medium in the amount 6-8 g per 100 ml.

On the medium containing phenols and aromatic hydroxy acids, the growth of polyporus mycelium was much less than that on medium containing sawdust, and the culture fluid very soon turned dark brown as a result of the formation of dark oxidation products. Darkening of the medium was usually accompanied by poorer growth of the fungus. Maximal production of the complex on media without sawdust, judging by the amount of substances precipitated by hydrochloric acid, was not later than after a month. Then a decrease in the amount of complex, with accompanying autolytic effects, was usually observed (Fig. 1). Therefore the experiments on media without sawdust were continued for not more than 1.5 months.

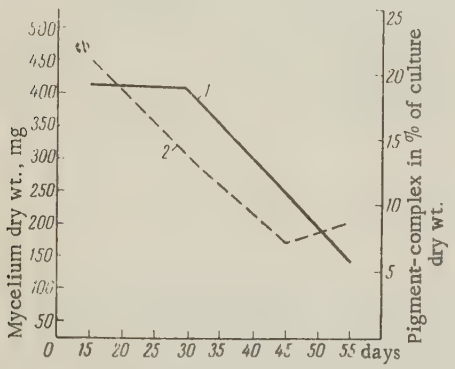

Fig. 1. Change in pigment-complex value during polyporus growth. 1) Pigment-complex; 2) mycelium dry weight.

On media containing sawdust, on the contrary, the amount of complex formed is directly related to the duration of the duration of the experiment over a long period of time, for many months. The amount of pigment-complex obtained from prolonged growth of the fungus was so large that it could be analyzed quantitatively, which could never be done in experiments on media without sawdust.

In beginning to describe the pigment-complex formed by polyporus in culture, it should be noted that the attempt to obtain the complex with so-called precursors has not for the present given the desired results. Thus, on the media with added phenols a complex is produced containing principally p-hydroxybenzoic acid. In the presence of syringic acid, the hydrolyzates contained vanillic and p-hydroxybenzoic acids and only traces of syringic. This indicates that the fungus removes the methoxy group from syringic acid. On medium containing vanillic acid, the complex contained p-hydroxybenzoic acid and traces of vanillic, again confirming that the fungus removes methoxy groups. The syringic and vanillic acid derivatives were broken down by the mycelium to syringic and vanillic acids. On medium containing rhamnose a very dark precipitate was obtained, whose hydrolysis products gave on the chromatogram no spots characteristic of the polyporus pigment complex.

From the media containing birch sawdust a pigment-complex was obtained, identical in qualitative composition and in quantitative ratio to that in the natural polyporus, on medium with sawdust from mature wood and on medium with sawdust after growth upon it of brown rot fungi. On the medium containing sawdust from young birch twigs, in spite of obtaining a large precipitate of pigment-complex resembling in appearance the precipitate from the natural one, the hydrolysis products of the precipitate contained very little methoxylated aromatic acids and considerably less than in the precipitate obtained on the regular sawdust (Table 2).

Table 2. Comparative Composition of Hydrolysis Products of Pigment-Complex Formed by Fungus in Artificial Culture on Mature and on Young Wood

Fraction	Mature wood	Young twigs
a	26,07	39,04
b	5,8	6,72
c	16,2	1,92
d	6,6	3,58
e	2,0	0,77

The results in the experiment on sawdust from young twigs can be explained by the fact that the lignin in young twigs is still in a state of formation, and besides the lignin there are products of incomplete condensation and just individual aromatic compounds.

The chemical composition of the precipitated complex, obtained on medium containing sawdust from mature wood, was investigated after 3, 6, 10, and 14 months of growth. Chromatography of the ether-soluble hydrolysis products of these complexes revealed nearly all the compounds characteristic of the natural polyporus. Quantitative study of the composition of the hydrolyzates confirmed the qualitative analytical data (Fig. 2). Thus, the composition of the precipitated pigment-complex of the polyporus culture 14 months of age is almost completely identical to that of the natural polyporus.

Such a length of time, however, is undesirable, and we therefore decided, in order to accelerate complex formation, to employ sawdust after previous growth upon it of brown rot fungi. These fungi, which utilize cellulose and break its bonds to lignin without affecting the latter, cause the lignin to become more available to fungi able to utilize it. This lignin has been termed biological lignin, since it is obtained by biological means rather than chemically.

Experiments on sawdust with the so-called biological lignin lasted 6 months. Below, in Table 3, are given the data from chemical analysis of the pigment-complex obtained on biolignin.

As seen from the table, the pigment-complex obtained on medium with sawdust containing biolignin is identi-

ble 3. Comparative Composition of Hydrolysis Products of Precipted Complex of Natural Polyporus and That Obtained on Biolignin

Fractions from silica-gel column	Complex on biolignin	Natural complex
A (unidentified acids)	26.14	25—35
B (pigments)	4.19	4—6
C (syringic acid)	15.16	15—17
D (vanillic acid)	5.05	5—7
E (p-hydroxybenzoic acid)	4.62	1—2
Total substances eluted from column	55.16	50—67

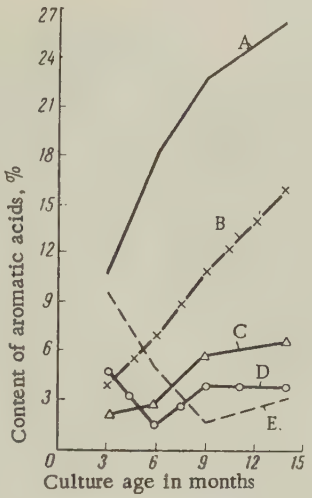

Fig. 2. Change in qualitative composition of fungus pigment-complex during growth in artificial culture. Data on dry weight of ether-soluble compounds in complex hydrolyzate. A) Unidentified acids; B) pigments; C) syringic acid; 3) vanillic acid; E) p-hydroxybenzoic acid.

al in composition to the polyporus complex produced nder natural conditions.

The two-fold acceleration in complex formation on ıedium with biolignin indicates that in order to utilize ıe lignin in ordinary wood the fungus must expend much work in liberating the lignin molecule from the wood.

The data presented in this study indicate it to be possible in principle to obtain polyporus pigment-complex under conditions of artificially culturing the fungus.

SUMMARY

1. In growing the polyporus fungus in artificial culture, media were employed containing phenols, aromatic acids, and methoxylated carbohydrates, and also sawdust, as possible sources for pigment-complex formation.

2. Formation of a complex identical in composition to that produced under natural conditions by the fungus was observed only on medium containing sawdust.

3. The use of sawdust containing so-called biolignin made it possible to cut in half the time for complex formation.

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ON THE USE OF ANTIBIOTICS FOR PROTECTING FIBROUS EXTERIORS OF UNDERGROUND POWER CABLES FROM DESTRUCTION BY MICROORGANISMS

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The loss of strength in the external fibrous cover of underground power cables due to microorganisms considerably reduces their period of service. The cable industry is at present employing naphthenate of copper to treat cellulose fiber (cable thread) for resistance to cellulose-decomposing bacteria, and oxydiphenyl for resistance to mold fungi. The use of these compounds, however, is not always properly effective or convenient. They have a sharp unpleasant odor and pollute the factory.

The work whose results are presented in this article is the first attempt to use antibiotics as antiseptics for cable thread.

METHODS

One of the authors (Zhukova, 1960) isolated a large number of antagonists to cellulose microorganisms from soil of the Kola Peninsula: 1947 bacteria, 1195 fungi, and 125 actinomycetes able partially or completely to inhibit the growth of Vibrio vulgaris, Sorangium cellulosum, and Sporocytophaga myxococcoides. In addition, 60 actinomycete cultures antagonistic to cellulose bacteria were obtained from the Division of Microorganism Interrelations, Institute of Microbiology AN SSSR. From these cultures were selected the microorganisms that most actively inhibit the growth of aerobic cellulose bacteria, anaerobic thermophilic cellulose-fermenting bacteria, and the fungus Trichoderma lignorum that actively decomposes cellulose. The antibiotic activity was tested in the culture fluid of the experimental cultures. Sporogenous bacteria were grown in meat-peptone broth, fungi on Czapek medium, and actinomycetes on fish medium. Glucose in the amount 2% was added to all media as carbon source. Cultures were grown on shakers at +27°. In addition to the action of culture fluid from the microbes antagonistic to cellulose-decomposing microorganisms, the action of antibiotics (biomycin, grisemin, penicillin, streptomycin, virusin, and preparations Nos. 108 and 1609) were tested on the same cultures. The antibiotics, except preparation 108, were used as aqueous solutions, 1 mg per ml distilled water. Preparation No. 108 was used as an alcoholic solution.

Experiments were set up under aerobic and under anaerobic conditions. In tests made under aerobic conditions, filter paper or cotton thread, saturated with antibiotic, was dried, placed in a Petri dish on Gerchinson agar medium or wort agar, moistened with nutrient medium, and inoculated with the aerobic cellulose bacteria V. vulgaris, Sor. cellulosum, or S. myxococcoides, or with the fungus T. lignorum. Plates were put in a moist chamber at + 27°. Control samples of paper and thread, untreated with antibiotic, were also moistened with nutrient medium and inoculated with the same cellulose-decomposing microorganisms. Growth of the microorganisms on the paper and thread was noted during the experiment. When the experiment was over the thread was removed from the plate surface, washed with water, dried, and its strength tested on an instrument for determining tensile strength (dynamometer). Tensile strength was also tested prior to inoculating the thread saturated with antibiotic with the cellulose-decomposing microorganisms. In the tests made under anaerobic conditions, cotton thread treated with antibiotic was immersed in nutrient medium previously inoculated with an enriched culture of anaerobic thermophilic bacteria (Imshenetskii, 1953). At the end of the experiment the thread was washed with water, dried, and tested for tensile strength.

RESULTS

Paper and cotton thread, treated with culture fluid from antagonistic sporogenous bacteria or fungi and then inoculated with aerobic cellulose bacteria, become more resistant to growth and action of the latter. In Fig. 1 is shown the destruction of cotton treated with Bacillus mesentericus 2699 culture fluid and inoculated with aerobic cellulose bacteria. Such cotton decomposes completely in the 30-day experiment, whereas the cotton treated with culture fluid of antagonistic actinomycetes loses only up to 40% of its original strength in this time. It is seen in Fig. 2 that on the cotton treated with Actinomyces lavendulae 4033 culture fluid there is no growth of cellulose bacteria. The experiments showed that the actinomycetes are more active antagonists to cellulose-decomposing microorganisms than the antagonistic fungi or sporogenous bacteria.

It is seen from Table 1 that after cotton and paper are treated with culture fluid from A. lavendulae 4033, Actinomyces rimosus, and Actinomyces fluorescens 2292 and 592, no growth of the aerobic cellulose-decomposing microorganisms is noted on the treated material. The treated cotton, inoculated with thermophils, retained its original strength for 10 days, whereas the untreated cotton was completely decom-

Fig. 1. Growth of aerobic cellulose bacteria on cotton thread treated with Bacillus mesentericus 2699 culture fluid and inoculated with Vibrio vulgaris, Sorangium cellulosum, and Sporocytophaga myxococcoides (30-day experiment).

Fig. 2. Absence of growth of aerobic cellulose bacteria on cotton thread treated with Actinomyces lavendulae 4033 culture fluid and inoculated with Vibrio vulgaris, Sorangium cellulosum, and Sporocytophaga myxococcoides (30-day experiment).

Fig. 3. Absence of growth of Trichoderma lignorum on cotton thread treated with biomycin (30-day experiment).

Fig. 4. Growth of Trichoderma lignorum on cotton thread untreated with antibiotic (30-day experiment).

Table 1. Effect of Culture Fluid from Actinomycetes on Growth of Cellulose-Decomposing Microorganisms

Cellulose treated with actinomycete culture fluid		Growth of aerobic cellulose-decomposition microorganisms			Growth of anaerobic thermophilic bacteria					
		growth on paper in 30 days		growth on cotton in 30 days	No. of days to full loss in strength of cotton inoculated with bacteria					
		sorangium cellulosum	sporocytophaga myxococcoides	trichoderma lignorum	4	5	6	7	9	10
Control (untreated)		+	+	+	+					
Act. lavendulae	4518	—	—	—	—	+				
" "	4033	—	—	—	—	—	—	—	—	+
" "	2335	+	+	+	—	+				
Act. rimosus		—	—	—	—	—	—	—	—	+
Act. fluorescens	2292	—	—	—	—	—	—	—	—	+
" "	592	—	—	—	—	—	—	—	—	+
Act. griseus. Kras	70	—	—	+	—	—	—	+		
" " "	15	—	—	+	—	—	—	+		
" " "	292	—	—	—	—	+				
" " Waks. Paryz.	1	+	+	+	—	+				
Act. violaceus	719	—	—	+	—	+				
Act. luridus	111	—	—	+	—	—	—	—	+	

Note. + signifies growth of aerobic cellulose-decomposing microorganisms; full loss of cotton strength; — no growth of aerobic cellulose-decomposing microorganisms; 100% of cotton strength retained.

posed in 3–4 days of the experiment. The actinomycetes enumerated above are the most active producers of antibiotics compared to the other organisms we tested. The titer of antibacterial activity in culture fluid from A. fluorescens 2292 and A. rimosus was 730 units towards Staphylococcus aureus; it was determined by the dilution method in meat-peptone broth. Titer of antibacterial activity of A. fluorescens 592 and A. lavendulae 4033 was 250 units.

The bactericidal action of the antibiotics was stronger on the aerobic cellulose-decomposing microorganisms than on the anaerobic thermophilic bacteria. There was no growth in 30 days of S. myxococcoides on paper treated with the antibiotics (Table 2). Sor. cellulosum and T. lignorum grew only on the paper treated with antibiotic No. 108. The various antibiotics protected the cotton from destruction by thermophils to a varying extent. Bacterial growth on cotton is weakly inhibited by grisemin and penicillin. Cotton treated with these antibiotics lost its strength only 2–3 days later than the untreated. Cotton treated with streptomycin, terramycin, virusin 1609, and antibiotic No. 108 decomposed under the action of thermophils in 10 days and that treated with biomycin retained its original strength for 15 days, whereas cotton not treated with antibiotic decomposed in 3–4 days. Tests were also carried out in soil. Untreated cotton and cotton treated with culture fluid or antibiotic were placed in chernozem at a depth 10 cm from the surface with soil moisture 60% of full moisture capacity and temperature + 20°. As seen from Fig. 5, cotton treated with actinomycete culture fluid or biomycin is twice as resistant to the action of soil microorganisms as the untreated. The untreated cotton loses 100% of its original strength in 15 days of testing, while the treated cotton under analogous conditions loses 100% of its strength in 30 days. The best results were obtained with cotton treated with A. rimosus culture fluid. This cotton has the greatest resistance to the action of soil microorganisms, full loss of strength occurring in 40 days and in the untreated cotton in 15 days.

Table 2. Effect of Antibiotics on Growth of Cellulose-Decomposing Microorganisms

Antibiotic	Growth of aerobic cellulose-decomposing microorganisms			Growth of anaerobic thermophilic bacteria					
	growth on paper in 30 days		growth on cotton in 30 days	No. of days to full loss in strength of cotton inoculated with bacteria					
	sorangium cellulosum	sporocytophaga myxococcoides	trichoderma lignorum	4	5	6	7	8	10
Control (untreated)	+	+	+	+					
Biomycin	—	—	—	—	—	—	—	—	—
Grisemin	—	—	—	—	—	+			
Penicillin	—	—	—	—	—	—	+		
Streptomycin	—	—	—	—	—	—	—	—	+
Terramycin	—	—	—	—	—	—	—	—	+
Virusin 1609	—	—	—	—	—	—	—	—	+
No. 108	+	—	+	—	—	—	—	—	+

For symbols see Table 1.

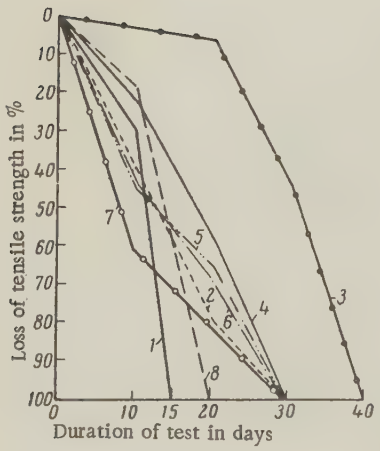

Fig. 5. Loss of tensile strength after soil tests in cotton cable thread treated with actinomycete culture fluid and with antibiotics. 1) Control (cotton treated with water); 2) cotton treated with Actinomyces lavendulae 4033; 3) cotton treated with Actinomyces rimosus; 4) cotton treated with Actinomyces fluorescens 2292; 5) cotton treated with Actinomyces fluorescens 592; 6) cotton treated with biomycin; 7) cotton treated with streptomycin; 8) cotton treated with virusin 1609.

SUMMARY

The strongest antibiotic effects towards cellulose-decomposing microorganisms are shown by the antibiotics biomycin, streptomycin, terramycin, and virusin 1609, and by culture fluids from the Actinomyces A. rimosus, A. lavendulae, and A. fluorescens.

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THE ANTIBIOTIC PROPERTIES OF IODINATED STARCH AND ITS COMPONENTS

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During the last quarter of a century medicine has been enriched by a number of antimicrobial agents. Together with their positive therapeutic effect in many infections they possess a number of undesirable properties: the absence of therapeutic effect with viral infections, the formation of resistant strains, the development of candidiasis, toxic, and allergic reactions, the suppression of immunologic reactions, etc.

A renewed search for sulfonamide and antibiotic agents in the laboratories of all countries bears witness to the fact that the antibiotics in current use do not fulfill all the requirements of clinical medicine. The newer representatives of these compounds do not justify the hope placed on them as they possess these very deficiencies.

The undesirable effects of all these preparations are explained by their chemical similarity.

Thus, it appears to be quite justified to conduct experiments to obtain new antimicrobial preparations of some chemical structure which might be devoid of these defects.

It seems to us that, in this regard, attention should be directed toward macromolecular compounds containing iodine.

Iodine posesses a universal antimicrobial effect but unfortunately it is very toxic for higher organisms. Furthermore the usual therapeutic forms as the alcohol distillate or Lugol's solution are completely unsuitable as antiseptics for internal administration. Iodine in this form is rapidly bound to protein in the tissues with the formation of inactive compounds. In combination with complicated organic molecules iodine usually loses its antimicrobial activity.

In view of this the nontoxic, stable, high-molecular-weight complexes in which iodine maintains its antimicrobial effect are worthy of attention. One of the most interesting polymers of this type is starch and its components, in particular amylose.

The color reaction of starch with iodine was discovered in 1814 by Colin and Gautier de Clobrie and, later in the same year, independently by Stromeyer.

An explanation of this reaction has been the subject of a large number of papers. In explaining the character of the iodine bond with polysaccharide molecules some authors considered the product of the interaction a true chemical bonding while others considered it an adsorption. Nezvedtsky (1934) first expressed the idea of a biphasic reaction of iodine with the polysaccharide. The first phase gives a true chemical bonding and ends when 38.4 mg of iodine combine with 1 g of starch or 53.4 mg of iodine combine with 1 g of amylose. Then the process of adsorption begins.

Freudenberg (1939) proposed a helical model for the structure of the complex: an amylose chain forms the spiral, inside of which, along the axis, the atoms of iodine are arrayed; upon completion of the spiral, iodine then combines by adsorption.

Further development of this theory is given in the work of Rundle and coworkers (1943) and by other authors.

Almost nothing is known of the antimicrobial effect of the iodine and polysaccharide complexes. Lumiere (1917) briefly reported that solubilized starch and iodine complexes posess antimicrobial properties and can be used in Dakin's solution in the treatment of infected wounds. This method has not been developed any further.

Iodine-amylose and iodine-amylopectin compounds have not been cited in the literature for their antimicrobial effect.

Mokhnach (1942) showed, independently from Lumiere, that colloidal iodinated starch posesses a therapeutic effect in the treatment of bacterial dysentery. These observations caused us, in 1956, to renew the study of antimicrobial and therapeutic properties of iodinated polysaccharides.

The results of our investigations on the antibacterial effect of iodinated starch (soluble), of iodinated amylose and iodinated amylopectin are presented in the present work.

METHODS

Solutions of iodinated starch, iodinated amylose, and iodinated amylopectin were prepared by the action on 0.2 to 1% polysaccharide solutions of a measured amount of potassium polyiodide (1 part by weight of I_2 to 2 parts by weight of KI in 100 ml of distilled water). The concentration of molecular iodine in various solutions varied from 0.027% to 1%; the concentration of iodinated polysaccharide was about 1%. Iodine chloride in the form of a 0.1 N solution was prepared by the method described by Gengrinovich (1946).

To study the antibacterial effect of the preparations, the solutions were diluted in saline. To each dilution

Table 1. The Antimicrobial Spectrum of Iodine-Polysaccharide Complexes

Test microbe	Concentration of iodine in which growth was completely suppressed (in μg/ml)		
	iodine-starch	iodine-amylose	iodine-amylopectin
1. Staph. aureus	3—10	2—5.4	10
2. Strept. haemoliticus	5—10	2.7	—
3. Escherichia coli	10	1.4—2.7	10
4. Escherichia coli 0-26; 0-111 enteropathogenic	10—20	2.7	—
5. Salm. typhiabdominalis	10	2.7	—
6. Sh. dysenteriae Sonne	20	5.4	—
7. Sh. dysenteriae Flexner	10	5.4	—
8. Bact. pyocyaneum	10	—	—
9. Proteus vulgaris	10	10.8	—
10. Bac. mycoides	10	1.4	—
11. Bac. mesentericus	10	—	—

Table 2. The Comparative Antibacterial Activity of Iodine-Amylose, Potassium Polyiodide with the Same Titre of Molecular Iodine

Test microbe	concentration of active iodine in gamma/ml				
Iodine-amylose					
Staphyl. aureus	16 (—)	8 (—)	4 (—)	2 (—)	1 (+)
Escherichia coli	16 (—)	8 (—)	4 (—)	2 (—)	1 (+)
Potassium polyiodide					
Staph. aureus	16 (—)	8 (+)	4 (+)	2 (+)	1 (+)
Escherichia coli	16 (—)	8 (+)	4 (+)	2 (+)	1 (+)
Iodine chloride					
St. aureus	2.5 (—)	—	—	—	—
Escherichia coli	2.5 (—)	—	—	—	—

Notation: (—) absence of growth, (+) presence of growth

of iodine-polysaccharide was added a suspension of bacteria in saline in an amount of 5 million cells prepared from 24-hour agar cultures of the test strain. After 15 minutes of exposure to each dilution, the bacterial suspension was planted on a meat peptone agar in Petri dishes. The results were read in 24 hours for the absence of bacterial growth which indicated complete bactericidal activity of the iodine-complex solution. The presence of isolated colonies of the test organism on the agar, with complete growth in the control plates, meant partial activity of the preparation.

RESULTS

The data given in Tables 1 and 2 show that the antimicrobial effect of iodinated polysaccharide is not only quite effective but in a number of instances significantly exceeds the effect of potassium polyiodide.

This observation deserves special attention in view of the fact that the polysaccharides under investigation —starch and amylose—by themselves not only posess no antimicrobial activity but also are used as nutritive material for the microbes. In commenting on such an increase in the antimicrobial effect of the iodinated polysaccharides it is necessary first of all to evaluate the antimicrobial effect of all the components of our solutions. In the polyiodide solution there are anions of iodine, molecules of I_2 polarized by the iodide ions and molecules of I_2 polarized by the solvent molecules. As the iodine anion does not have antimicrobial action (the aqueous solutions of iodides of alkali and alkaline earth metals are inactive as regards bacteria), the antimicrobial effect of polyiodide solutions might be ascribed to the polarized molecules of iodine.

Iodine forms, in the presence of starch or amylose, compounds of intense blue color. If one holds to the theory of the structure of these compounds which was proposed by Freudenberg (1939) then the antimicrobial effect of the iodine-polysaccharide compounds might be explained by the following hypothesis. Tens and hundreds of I_2 molecules combined with the polysaccharide form single molecules with the shared electrons attracted, to the greatest degree, to the highly polymerized compound. Because of the fluctuation in electron density in these compounds, the latter can become donors of cations. Iodine cations have strong oxidizing properties which explains their antimicrobial effect of such broad spectrum.

To substantiate this, experiments were carried out to study the antibacterial effect of solutions of iodine chloride. Such solutions undoubtedly contain iodine in the form of the cation I^+ and have powerful antimicrobial effect (Table 2). This effect can be ascribed to the action of the iodine cation as the chloride anion as well as the iodine anion posesses no antimicrobial effect.

As is seen in Table 1, the iodinated starch and amylose have a broad spectrum of antimicrobial effect. They have the same high bactericidal effect on both the gram-negative and gram-positive organisms. The bactericidal effect is observed in iodine concentrations of 2.5 to 20 μg/ml after 15 minutes exposure. Suppression of growth is observed with smaller concentrations. The iodinated amylose is more bactericidal than the starch.

The antibacterial effect of the iodine-amylopectin complex was investigated with Staphylococcus aureus and Escherichia coli. Complete absence of growth in this instance was observed with an iodine concentration of 10μ g/ml.

The antibacterial action of iodine chloride was also investigated with S. aureus and E. coli. An effect was achieved with an iodine concentration of 2.5 μg/ml.

The effect of alkali on solutions of iodine-polymer complexes resulted in instantaneous discoloration. In contradistinction, the discoloration produced by heating can not be restored. As our experiments showed, the antimicrobial activity of the iodine-polysaccharide complex is completely lost after discoloration by alkali. Apparently the effect of alkali is the destruction of the staining and antimicrobial centers, formed by the cations of iodine or by the polarized iodine molecules. During alkali treatment an alkaline metal iodide is formed in which iodine is in the form of an anion—colorless and devoid of antimicrobial activity.

SUMMARY

1. Starch iodine, amylose iodine and amylopectin iodine, have a wide antibacterial spectrum.

2. While being incorporated into the polysaccharide molecule, iodine does not exhibit any reduction of its antibacterial action. Moreover, it exhibits even an increase in its antibacterial properties which is particularly apparent in amylose iodine.

3. The antimicrobe action of polyiodine solutions may be attributed to polarized iodine molecules.

4. The high antibacterial activity of aqueous solutions of iodine chloride is due to iodine cation.

5. The antibacterial properties of solution of iodine-polysaccharides are completely impaired upon decolorization by alkali.

6. In colored iodine-polysaccharides as well as in other colored iodine compounds the center of staining possessing antimicrobe action is the iodine cation or the polarized iodine molecule. The iodine anion is colorless and does not possess any antimicrobe activity.

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DISCUSSION

ON THE DIAGNOSTIC SIGNIFICANCE OF VARIOUS CHARACTERISTICS FOR CLASSIFICATION OF REPRESENTATIVES OF THE GENUS ACTINOMYCES (STREPTOMYCES)

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Intensive search for antibiotics directed attention of many investigators to actinomycetes. The development of principles for classification of actinomycetes is, consequently, of considerable theoretical, as well as practical, significance.

Many new species of actinomycetes have been described during the past few years, and it became necessary to develop a definitive scheme for their classification based on their most important constant and permanent characteristics. The timeliness of this matter was stressed by many investigators during the symposium on taxonomy of actinomycetes in Stockholm in 1958.

It is entirely natural for the investigators to wish to establish as the basis for classification not occasional, but constant, characteristics connected with basic physiological activity of the cell. This is an exceedingly complex subject since there are only a few diagnostic characteristics in the actinomycetes. Cytologic and genetic studies are in the initial stages of development in actinomycetes, and although of considerable help in classification of other organisms, they are not, at this time, useful for solving these problems for actinomycetes.

The morphology of sporophores and spores, pigmentation of the substrate and aerial mycelium, soluble pigments, and physiological and biochemical properties are included in characterization of the species in the existant keys to actinomycetes (Krasil'nikov, 1949, Waksman, 1953) and in description of species in the current journals.

Many investigators are confronted in their studies with considerable variability of actinomycetes in which case the series of heretofore useful taxonomic characteristics becomes unstable and useless for all practical purposes. During 1954–1959 in the Institute for Investigating New Antibiotics, the authors studied, in this connection, the stability of cultural and morphological characteristics of 471 cultures of actinomycetes of different species. Stability and significance of physiological (biochemical) properties in taxonomy of actinomycetes will be discussed in a separate article.

METHODS

Observations on stability of characteristics with time were made using Gauze's medium #1. This medium has been selected by us as being best suited for growth and development of actinomycetes. The strains were transferred twice a year throughout this study. In transferring, one paid attention to stability of cultural characteristics. Sporophore morphology has been studied twice—at the start of the investigation in 1955 and again in 1959.

MORPHOLOGY OF SPORANGIA AND SPORES

As indicated by Krasil'nikov (1949), Pridham, et al., (1958), Waksman (1957), and others, the structure of fruiting organs, sporophores and spores, is an important characteristic in taxonomy of actinomycetes. Recently completed detailed studies of sporangial morphology, carried out by Pridham, et al. (1958) and Ettlinger, et al. (1958), allowed the establishment of different forms of spirals, clarification of the nature of branching of sporophores, and in this manner added to our knowledge of actinomycetes. In analyzing sporophore morphology, it is necessary to differentiate clearly between three concepts. These are: the nature or type of sporangia (spiral or straight), their arrangement (single, multiple, and so forth), and the nature of branching (monopodial, broom-shaped, sympodial). Therefore, one cannot contrast straight and spiral sporophores with broom-shaped, although this is often done.

A study of stability of sporophore morphology (Table 1) indicated that this characteristic is constant in the aerial mycelium with normally developed sporophores.

Changes in nature of sporophores (most frequently encountered—disappearance and reduction in the number of coils) were observed in the strains that lost, as a result of transferring, the ability to produce normal aerial mycelium.

Unfortunately, there are difficulties and exceptions in subdividing actinomycetes on the basis of sporophore morphology.

Table 1. Stability of Morphological and Cultural Characteristics of Actinomycetes During a Four-Year Period of Laboratory Propagation

Actinomycetes grouped according to aerial mycelium	No. of species	No. of strains	Of these, following retained: sporophore morphology	pigmentation of aerial mycelium	pigmentation of vegetative mycelium	No. of strains with soluble pigment	Of these soluble pigment retained in
White, pale yellow	20	81	75	81	73	64	58
Rose	26	89	89	89	84	15	9
Gray	42	235	232	235	187	67	53
Blue	8	66	64	64	52	15	2
Total	96	471	460	469	396	161	122
%			97	99	84		76

Attempts to divide the strains in our collection into morphologic sections, according to the decision of the Stockholm conference of 1958, indicated that it is often quite difficult, and probably not advisable, to separate members of Rectus from those of Rectus flexibilis. Therefore, according to Pridham's recommendation, it appears to us that the straight and the wavy sporophores must be placed in the same category.

Similar difficulties are encountered in separation of Retinaculum apertum from strains with spiral sporophores. Nevertheless, it appears to us that it is useful to study the morphological details of coiled sporophores. The existence of tightly wound, closed coils is a highly constant characteristic of certain species. Among actinomycetes there exist the so-called 'paired' species and varieties which are differentiated only by sporophore morphology. To these belong Actinomyces violaceus and A. violaceus var. rectus, Actinomyces flaveolus and A. flaveolus var. rectus, Actinomyces fradiae and A. fradiae var. sporalis and others. Among the producers of aureomycin we observed that strain 3894 formed straight sporophores even though it could not be differentiated from Actinomyces aureofaciens either by means of the most important cultural characteristics or production of the antibiotic. The spirals were absent when this strain was also grown on other media (Czapek, Krasil'nikov—CP-1, Lindenbain). Examples of cultures of actinomycetes strongly resembling each other in cultural, physiological characteristics and formation of antibiotic (indicating similarity of metabolic activity), and differentiated only by sporangial morphology, are not exceptional in nature.

In studying cultures of certain species such as Actinomyces litmocidini, Actinomyces chinensis, and others, we found both straight and coiled sporangia.

In certain cases, by streaking cultures with coiled sporangia, one can isolate colonies retaining all the cultural characteristics of the original culture, but possessing straight sporangia. Straight sporangia were retained in transferring. These observations were made by Svershnikova in A. chinensis.

From the above, it is apparent that it is not always possible to consider the structure of sporangia to be of primary significance. We believe that it is not desirable to combine species into large groups on the basis of this characteristic, since, in this case, closely related species and varieties will be placed in entirely different categories.

SPORES

New, interesting and important additional data pertaining to spores of actinomycetes have been obtained by means of electron microscopy. Several investigators (Kustor, 1953, etc.) established the existance of three types of spore surfaces: smooth, spiny, and hairy. According to the results of Ettlinger and his coworkers (1958), as well as on the basis of our data, the structure of spore surface is reasonably stable in nature. According to Maksimova, Kudrina, and Mordarski, modification of sources of carbon and nitrogen do not result in marked changes in the nature of growth on spore surface.

Analogous results were obtained in examination of different age cultures. Data are available pertaining to the existence of established differences between species, based on the extent of growth on spore surface. It has also been shown by means of electron microscopy that the size and shape of spores are variable in many species, and, consequently, these characteristics are not of diagnostic significance for most species.

Although spore morphology of actinomycetes is a constant characteristic, it is limited in diagnostic significance inasmuch as the spore surface growth is by no means a property of all species of actinomycetes. Most species have smooth spores.

AERIAL MYCELIUM

The pigmentation of aerial mycelium is considered by many investigators as one of the basic diagnostic characteristics (Burkholder, 1954, and others).

The natural classification of higher and lower organisms is based on the knowledge of the morphologic peculiarities of fruiting bodies. The characteristics embrace not only their morphology, but also their external appearance, dimensions, pigmentation, etc.

From our point of view, the pigmentation of sporebearing aerial mycelium of actinomycetes is one of the characteristics of the organs responsible for multiplication of actinomycetes, and consequently is of equal importance to morphology for classification of actinomycetes.

Spore coloration is a more general macrocharacteristic of reproductive organs of actinomycetes; it must precede their microcharacteristic: the detailed investigation of sporophores and spores. Here it must be stressed that we have in mind sporebearing aerial mycelium and that we do not attribute diagnostic importance to sterile aerial mycelium.

Table 2. The Relation Between Pigmentation of Aerial Mycelium and the Nature of Spore Surface

Actinomycetes grouped according to aerial mycelium	Average No. of species	Number of species with smooth surface	spiked surface	hairy surface
White, cream and paleyellow	24	24	0	0
Rose and lilac	27	23	4	0
Gray	54	33	15	6
Azure and azure-green	9	0	7	2
Total	114	80	26	8

The results of an internationally carried out experiment on determination of different characteristics in actinomycetes show that the most constant properties are represented by the structure of spore surface and formation of melaninlike pigment, with the second and third places being relegated to the structure of sporophore and pigmentation of aerial mycelium. Pigmentation of the substrate mycelium is more variable and occupies the last place among these characteristics.

Studies on the aerial mycelium during 4–5 years of transferring of cultures on Gauze's #1 synthetic medium revealed the stability of blue, blue-green, gray, pale yellow, and rose-lilac pigments during this time (Table 1). These are well-preserved in 99% of the investigated cultures, providing the media are favorable for normal development of actinomycetes, and only rose-lilac pigment becomes paler. Changes in pigmentation of aerial mycelium are usually related to degeneration of a culture or to its inadequate development. Synthetic media proved to be best suitable for formation of sporebearing aerial mycelium; of these glucose-aspartic agar and Czapek's agar were least desirable. More detailed discussion about selection of diagnostic media will be considered in a separate article.

Data of electron microscopy of spores of large groups of actinomycetes, with different pigmentation of aerial mycelium, showed the existence of positive correlations between the pigmentation of aerial mycelium and the peculiarities of structure of the surface membrane (Table 2).

Thus, for example, actinomycetes with white and pale yellow aerial mycelium have only smooth spores. The overwhelming majority of species of actinomycetes with rose-colored aerial mycelium also have smooth spores and only four species develop growth on the spore surface. In contrast to this, the overwhelming majority of actinomycetes with blue aerial mycelium have spiny spores, and actinomycetes with blue-green aerial mycelium have only hairy spores. Our data pertaining to this point confirm those of Ettlinger, et al. (1958).

According to Sveshnikova and Maksimova's data dealing with the ability of certain species of actinomycetes to utilize different sources of carbon, there exist group differences between the gray and rose actinomycetes in regard to assimilation of sugars (Table 3).

Thus actinomycetes with grey aerial mycelium form a very active group characterized by ability to utilize actively all the sugars used in the experiment, while the rose actinomycetes are not nearly as active and can well assimilate only arabinose and utilize xylose and rhamnose in moderation.

In this manner, division of actinomycetes into large groups on the basis of pigmentation of aerial mycelium, initially determined by us purely on empirical grounds as a practically convenient characteristic, appears to be tied to the existing morphologic and physiologic differences between these groups.

SUBSTRATE MYCELIUM

Pigmentation of the substrate mycelium and the medium is dependent on formation of different pigments inside the mycelium, and their secretion into the surrounding medium. In classification of other organisms, for example algae, pigments form one of the most important characteristics for their division into classes. Unfortunately, very little is known about the pigments in actinomycetes, and studies in this direction are urgently needed. Nevertheless, we know from experience that the ability to produce pigments is relatively stable in some, and is well maintained in transferring; cultures producing such pigments are easily differentiated. Pigments responsible for violet, red, blue, brown colors are stable, while yellow, green, rose, and a mixture of red and brown are less stable. These pigments are lost frequently or entirely in preservation of cultures, or become less pronounced. A relatively large percent of strains that lost pigmentation (Table 1) occurs in cultures with yellow and green pigments. Perhaps this is related to the use of media poorly suited for formation of pigments. The development of pigment depends to a considerable extent upon the composition of a medium. Our preliminary data on utilization of Tresner's medium as modified by Koreniako, for green and yellow pigments, showed in a series of cases that there is stronger pigment production in this medium.

The presence of even such poorly preserved pigments helps the investigators to establish the taxonomic position of freshly isolated cultures which have, as a rule, brighter pigmentation.

Table 3. Utilization of Different Carbon Sources by Rose and Gray Actinomycetes

Actinomycetes, grouped according to aerial mycelium	Average No. of strains	Of these following utilize (in % of average number of strains in the group) rhamnose	fructose	xylose	raffinose	arabinose	mannose
Rose and lilac	178	34	26	45	24	63	23
Gray	138	72	65	61	32	69	60

A study of stability of coloration of substrate mycelium showed (Table 1) that 16% of the strains studied by us produced a change in color in starch and in nitrate media (most suitable for developing the typical pigmentation in substrate mycelium).

In establishing the pigmentation of substrate mycelium one must consider that upon storage of cultures the colorless substrate mycelium often acquires a straw-yellow, gray, weakly brownish tint. One should disregard these hues in classification of cultures.

Production of melanoid-like pigment is highly characteristic of actinomycetes. The property of actinomycetes to stain organic media brown is relatively constant in nature. However, the media used for this purpose, such as nutrient agar, MPA and peptone agar with bactotryptone and bactoyeast extract, are not very favorable for growing actinomycetes. In our opinion, Gauze's organic medium #2 is more suitable for this purpose.

Taking into consideration that, according to Baldacci (1959), the most important metabolic processes are localized in the substrate mycelium, it appears to us that its nature is of considerable importance, regardless of certain instability of its pigmentation. Moreover, the existence of color variants of the substrate mycelium, helps to differentiate species.

ANTIBIOTICS

Antibiotics, as pigments, are metabolic products of actinomycetes. Although in a great majority of cases, each antibiotic is produced by cultures of a definite species, we often are confronted with the fact that one and the same antibiotic is produced by different species, or that one species simultaneously produces several antibiotics. In addition, formation of an antibiotic is frequently unstable, and without proper selection, cultures lose their antibiotic properties. Because of these conditions we relegate, at this time, the formation of antibiotic to subsidiary and not to basic properties, although it is entirely possible that different species of actinomycetes, forming one and the same antibiotic, form different variants of the same agent. At the present, this is an open question, and additional investigations are necessary in this area.

PHAGE SPECIFICITY

According to Velsh (1958), Rantenshtein (1959) and other investigators, the available data dealing with application of specific phages for identification of species, allow utilization of this characteristic in taxonomy of actinomycetes, as an additional characteristic since, to date, only a few specific phages have been isolated.

DISCUSSION

The characteristics described above allow us to conclude that the following belong to the more stable criteria useful for describing the species of actinomycetes: pigmentation of spore-bearing aerial mycelium, spore, and sporophore morphology, pigmentation of the substrate mycelium, and of the dissolved pigments. In our opinion, formation of antibiotics, phage specificity, physiological and biochemical reactions (hydrolysis of starch, inversion of saccharose, reduction of nitrates) and others. Many investigators working on taxonomy of actinomycetes concluded independently that the species may be grouped into "series" and "sections," uniting similar organisms.

Recently several classifications of this type have been proposed by Waksman (1953, 1959), Baldacci (1954, 1959), Gauze, et al. (1957), Pridham, et al. (1958), Ettlinger, Corbaz, and Hutter (1958), Shinobu (1959) and other. They are all based on combination of several characteristics which are considered to be most important by almost all investigators. To these belong morphology of spore surface and sporophore, pigmentation of aerial and substrate mycelium, physiological properties. The authors differ among themselves only in which of these characteristics to consider important in unification of species.

It is highly desirable that the species be grouped according to basic and important common characteristics. From our data, the following belong to them: morphology of sporophore and spore surface and pigmentation of aerial and substrate mycelium. They are quite stable and in general are well-preserved in time.

At present it is still difficult to offer complete scientific substantiation for superiority of any one characteristic for grouping of species into series of categories; therefore, it is expedient to select characteristics of greater practical convenience.

Baldacci (1959) points out that it is necessary to consider all earlier publications, and that it would be incorrect to omit studies about actinomycetes carried out prior to 1919. From this point of view, the morphology of sporophores is not suitable for a classification which would attempt to encompass all heretofore-described species. We agree with Baldacci and can point out another limitation regarding combining of species into groups according to sporophore morphology, namely, the existence of closely related species and varieties that are differentiated only by sporophore morphology.

Baldacci proposes to combine species of actinomycetes into divisions depending upon the presence or absence of pigmentation of substrate mycelium. Within a division he differentiates groups according to pigmentation of substrate mycelium and then separates these into series according to pigmentation of aerial mycelium.

As indicated above, there is certain experimental evidence (electron microscopy, utilization of different sugars) for grouping actinomycetes according to pigmentation of sporebearing aerial mycelium; in addition this is a more constant property than pigmentation of the substrate mycelium. Consequently, we propose to subdivide actinomycetes of the genus Actinomyces (Streptomyces) into four large groups according to pigmentation of aerial mycelium into four large groups and to call these sections:

1. Section Niveus with white and pale yellow aerial mycelium,

2. Section Roseus with lilac and rose aerial mycelium,

3. Section Azureus with azure and azure-green mycelium,

4. Section Civereus with grey aerial mycelium.

It seems advisable to group the genera of actinomycetes into series according to the pigmentation of substrate mycelium, considering that it is the site of basic metabolic activity in actinomycetes and that its characteristic offers an opportunity to differentiate relatively easily between the component species.

The series of actinomycetes proposed by us above are divided into sections in the following manner:

Section Niveus—series Albus, Albosporeus, Helvolus.

Section Roseus—series Lavendulae-reseus, Fradiae, Ruber, Eusaid, Roseoviolaceus.

Section Civereus—series Criseus, Aureus, Thrysomallus, Violaceus, Chromogenes.

Section Azureus—series Coerulescens.

SUMMARY

From a study of cultural and morphological characteristics of 471 actinomycetes it follows that the most stable characteristics are the morphology of spores and sporophores, color of the air and substrate mycelium, and part of the soluble pigments. These characteristics can be related to the fundamental diagnostic ones. The formation of antibiotics, the physiological (biochemical) properties, and phage specificity are regarded as additional characters. It is suggested to divide actinomycetes according to the color of the sporogenous air mycelium into sections with further division into series according to the color of the substrate mycelium.

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BOOK REVIEWS

Microorganisms and Increase of Pond Fish Production, by A. G. Rodina
Press of the Academy of Sciences, USSR, Reviewed by M. V. Ivanov
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The appearance of a separate book with such a promising title and written, moreover, by an author so well known in hydrobiology and microbiology as A. G. Rodina, will inevitably have a wide appeal both for the numberous scientific workers engaged in research on the biology of aquatic organisms and for practical workers in pond fish farms, fishery collectives and fish-breeding farms. An excellent groundwork for the appearance of such a book has been laid by the whole course of development of aquatic microbiology in our country. The wide application of the direct-count method devised by Kholodnyi, Razumov, and other research workers, the all-around approach to the study of life in waters and the combination of field and experimental studies are what distinguishes the development of aquatic microbiology in the USSR. All these features of Soviet aquatic microbiology have enabled our microbiologists and hydrobiologists to interfere deliberately in biological production processes in waters, such intervention consisting mainly in the application of various organic and mineral fertilizers. The scope of such work has been very broad in the postwar period, and hence the publication of a book aimed at generalizing the information gained by various research workers in the field of microbiology of fertilized waters must be regarded as very timely and topical.

The choice of title of the book is not at all a happy one, since Rodina writes about fish production in particular only in two places (pp. 47 and 156–157), and there she confines herself to citing some very meager data on the increase of production in several ponds as a result of the application of fertilizers. More appropriate to the content of the book would have been the title which the author uses in the foreword and conclusion of her book: The Microbiology of Fertilized Ponds.

No great objections can be raised to the general arrangement of the material in the book; perhaps the places of chapters 2 and 3 should have been interchanged, since it would have been more logical to begin with the basic data on the food value of microorganisms and then to proceed to an account of the factual material relating to the increase of this type of food in fertilized waters. Incidentally, this was the historical sequence of the development of the thought of research workers, and of that of A. G. Rodina herself in the first instance.

The first chapter "Knowledge of microorganisms in the water and bottom of ponds. Physicochemical and biological factors of the medium and their importance for the development of bacteria in ponds", essential in a book published in a popular science series, is well written, illustrated with numerous figures, and introduces the reader straightaway to the range of questions dealt with in the book. One thing that should be mentioned is that no reference is made either in the first chapter or in the rest of the text to the importance of aquatic fungi of the group Oomycetes, some of which are parasites and have a considerable effect on fish production by causing mortality in the eggs and diseases in the fish. On the other hand, some fungi are important indicators of organic pollution of the waters, a factor which must also be taken into account in planning the introduction of organic fertilizers into waters.

The second chapter contains information on the bacterial content of the water and bottom deposits of ponds, and also discusses the question of the effect of different kinds of fertilizers on the abundance of the bacterioplankton and bacteriobenthos. A great fault of this chapter and, moreover, of the entire book is the persistent ignoring of the great amount of factural material obtained by the combined expedition of the All-Union Scientific Institute of Fishing and Oceanography, the Institute of Microbiology and Sevkaspribvoda in the fishery collectives of the Volga delta. A reader with inadequate background might gain the impression that the question of using hard aquatic vegetation as green manure has either not been considered at all by research workers, or has given such adverse results that even the mention of them is horrible.

One must inevitably come to such a conclusion on a reading of Rodina's book, where in 165 pages of text this method which, incidentally, is widely employed in productive practice, receives only one reference on p. 48, where it is stated that scything of reeds on large areas leads to a great reduction in the oxygen content of the water and to the suffocation of fish.

A second fault of this chapter is that the author concentrates her attention on the way fertilizers affect the magnitude of the bacterial biomass, without citing any figures on the production of bacterial biomass (data which are available in microbiological works including those cited in the bibliography), or even raising this question.

The third chapter—"Food value of microbes for aquatic invertebrates"—is the most successful in the book. This chapter gives a fairly full account of the biochemical data which underline the nutritive value of microorganisms, and cites a great deal of factual information obtained by various research workers and illustrative of the role of microorganisms in the feeding of various organisms dwelling in the water.

The fourth chapter—"Role of microorganisms in the circulation of matter in fertilized ponds"—occupies almost half of the book and causes some perplexity both in regard to the cited, or rather not cited, data

in it, as well as in regard to the interpretation of the obtained results.

A book dealing with the productivity of waters cannot be regarded as complete if the circulation of the very important organogenic element—carbon—is given only five pages (section entitled "Decomposition of nitrogenless substances"), while ten pages are devoted to the circulation of iron, for instance, and more than 40 to nitrogen. This is all the more inadmissible in that the main question examined in the book is the use of plants as fertilizer, which consist more than 90% of "nitrogenless substances". The book contains many vague phrases about the utilization of "dissolved organic matter" by bacteria, while the vast body of facts, obtained by Egorova and Deryugina on fish farms of the Volga delta, on the development of cellulose, pectin, and other bacteria fermenting and oxidizing carbohydrates on green fertilizer is left entirely out of the book.

Apart from some fragmentary data on the numbers of Azotobacter and yeasts there are no facts at all to illustrate the development of the various physiological groups of bacteria on the vegetation introduced as fertilizer, although the results of the same expedition to the Volga delta, ignored by the author of the book, showed that the numbers of bacteria in the decomposing vegetation could reach hundreds of billions of cells per gram, while the numbers of bacteria of the different physiological groups are numbered in hundreds of millions.

No attempt at all is made in the book to assess quantitatively the effect of microorganisms on the physicochemical conditions of fertilized waters. The total absence (from the book, and from the author's experimental work on which the book is mainly based) of elementary chemical determinations, such as the content of dissolved oxygen and hydrogen sulfide in the water, leads, in particular, to a wrong idea of the causes of such an important and serious effect in fish-rearing practice as the occurrence of suffocation conditions, which sometimes arise when fertilizers are employed unskillfully and unscientifically. If we are to believe Rodina's book (pp. 123–126), the main evil is the development of sulfate-reducing bacteria, leading to the formation of hydrogen sulfide—a phenomenon which is, in fact, extremely rare in fresh waters. At the same time the very real possibility of an oxygen deficiency occurring, particulary at night, owing to the rapid development and respiration of bacteria and phytoplankton in the region where fertilizers are introduced, scarcely receives a mention in the book.

All these major faults considerably diminish the value of the book, which falls far short of illustrating the present state of the treated subject.

One is particularly surprised at the contradiction between the complaints of the author on the inadequate treatment of several questions and her failure to give objective consideration to the numerous facts obtained by other teams of investigators.

CONFERENCE ON THE EXPERIMENTAL OBTENTION OF USEFUL VARIANTS OF MICROORGANISMS

Yu. N. Karasevich

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A Conference on the Experimental Obtention of Useful Variants of Microorganisms, convened at the suggestion of the Institute of Microbiology in order to coordinate research in this field, was held during January 21–23, 1960 in Moscow. The aim of the conference was to discuss the state of selection work in microbiology and the future lines of research on the obtention of microbial variants of economic value. Representatives of scientific research institutions of the Academy of Sciences, USSR, the Academies of Sciences of the Union Republics, the Academy of Medical Sciences, and the Academy of Agricultural Sciences, as well as representatives of branch institutes and industrial bodies—a total of 74 institutions—took part in the work of the conference.

The participants in the conference heard and discussed 23 papers.

Opening the conference, A. A. Imshenetskii stated that the best form of research coordination was the holding of topical conferences, at which not only the results obtained over a particular period of time could be discussed, but the lines of further research could be sketched out. Eight years had elapsed since the All-Union Conference on Directed Variation; during this period certain work had been carried out and it was now essential to sum up the results, and to reveal what methods gave the best results.

In the paper "Theoretical principles of selection of useful forms of microorganisms" A. A. Imshenetskii (Institute of Microbiology) pointed out that the two most fruitful and widely employed methods of selection were the adaptation of microorganisms to new conditions of life and the production of variants by the action of radiation and chemical substances. All the original (wild) strains of practically valuable microbes are isolated from natural substrates. Seeking and choosing the most active cultures from nature was the task of analytical selection. Only the most active wild cultures should be subjected to further experimental selection. For successful experimental selection it was necessary to expand theoretical research, in particular to engage in the search for the optimum doses of mutagenic factors and to study in detail the metabolism of the obtained variants in order to be sure of obtaining the required forms.

K. V. Kosikov (Institute of Genetics) in a paper entitled "Hybridization of microorganisms and its value for obtaining useful forms" mentioned that, as had been established recently, the sexual process was a widely occurring phenomenon among microorganisms. Recombination in bacteria and heterokaryosis in Fungi imperfecti could be regarded as special and more primitive forms of the sexual process.

Studies on hybridization had broadened and enriched our theoretical concepts of heredity and variation in microorganisms. In several cases hybrids of value to industry had been obtained. The continuation of research on hybridization must be regarded as very promising, and hence worthy of serious attention.

The paper of S. I. Alikhanyan (All-Union Institute of Antibiotics) dealt with the question of the use of physical and chemical factors in microbial selection. A deep theoretical analysis of the results of employing these factors was given in the paper.

S. I. Alikhanyan pointed out that almost all the present-day industrial cultures of antibiotic-producers had been obtained by the use of physical and chemical mutagenic factors, or by the combined action of these factors (for instance, the action of ethyleneimine and UV-rays). The paper outlined the trends of further research in this field.

"Results and perspectives of employing the method of continuous improving selection of yeasts from industry" was the title of a paper by V. I. Kudryavtsev (Institute of Microbiology). The reader showed that the use of continuous improving selection of industrial microorganisms held great promise for the alcohol, brewing, baking, dairy, and other branches of industry.

Examples of the successful employment of this method in several microbiological industries were cited.

In the paper of M. I. Sokolov (Institute of Virology) "Principles and methods of obtaining live vaccines" it was pointed out that the last twenty years had been distinguished by great achievements in the obtention of several new live vaccines against diseases of man and animals. The successes achieved in this field were a consequence of the application of materialist concepts of heredity and its variation to microbiology and virology. Various methods (physical, chemical, and biological) were employed for obtaining live vaccines.

The most effective was the use of biological methods of experimentally altering microorganisms, their adaptation to appropriate nutrient substrates, experimental animals, chick embryos, and tissue cultures.

Further success in the obtention of live vaccines depended on a study of hereditary factors and the mechanisms of their transmission, a deeper knowledge of the nature and mechanism of action of the external environment on heredity and its variation, further investigations on the factors of virulence and immun-

ogeny of vaccines, and the discovery of the conditions in which they are most effective.

The paper of E. I. Krasnikov and A. G. Grinevich (Academy of Sciences, Uzb. SSR) gave an account of the main results of research on the breeding of fermentative microorganisms for the industries of Central Asia.

Reference was made to the successful production of industrial strains of alcohol yeasts, as well as of lactic acid bacteria for fodder ensilage, by the use of various radiations.

R. D. Zubkova (Academy of Sciences, Kaz. SSR) in a paper entitled "Experience of employing the method of continuous improving selection of yeasts from industry in the Alma-Ata champagne factory" reported on the efficacy of this method for obtaining industrial yeast strains.

The successful obtention of new industrial strains of sherry yeasts by the adaptation method was reported on in the paper of N. F. Saenko (All-Union Academy of Agricultural Sciences) "Methods of selecting sherry yeasts for alcohol tolerance". The training of sherry yeasts to increased alcohol concentration was most successful in the continuous cultivation of yeasts in periodically renewed medium with the alcohol concentration being slowly and steadily increased from 14 to 18%.

In the paper "Molds of the genus Aspergillus flavus as active producers of proteolytic enzymes" by E. Ya. Kalashnikov and T. I. Trainina (Ukrainian Institute of the Food Industry) it was stated that A. flavus fungi clearly distinguish themselves from other species of molds by their ability to accumulate a large quantity of proteolytic and a relatively small quantity of amylolytic enzymes in the culture. A test of the investigated cultures in industrial conditions showed their good qualities.

E. S. Tatarenko, I. P. Gerasimova, and I. P. Ternikova (Ukrainian Institute of the Food Industry) gave an address on the theme "Variation of experimentally obtained forms of the fungus Aspergillus oryzae". The variant No. 476 "N", obtained by UV-irradiation, possessed high amylolytic activity. It has been introduced into practice in several factories. The authors observed parasitism in a single spore culture of variants of the original stock form A. oryzae No. 476.

M. I. Prokhorova (Academy of Agricultural Sciences) in "Some properties of mouselike-rodent typhus bacteria No. 5170, obtained experimentally from the colon bacillus", reported that these bacteria were obtained by forcing the colon bacillus (in vitro) to assimilate substrates consisting of killed rodent typhus bacteria (Bact. decumanicidum) and sterile extracts of organs of grey rats.

R. A. Menkina in the paper "Effect of selection on some physiological properties of Bacillus megaterium var. phosphaticum pointed out that in the production of phosphobacterin the rate of growth of the culture and the rate of spore formation were of great importance for the avoidance of phagolysis. For this reason constant selection of these cultures was necessary.

Workers in the All-Union Institute of Antibiotics presented papers in which they cited examples of the successful obtention of industrial strains of antibiotic- and vitamin-producers by means of physical and chemical mutagenic factors.

The following comrades spoke: F. S. Klepikova (All-Union Institute of Antibiotics) "Regular features of variation in Actinomyces subtropicus under the action of ionizing radiations", N. I. Zhdanova (All-Union Institute of Antibiotics) "Use of ethyleneimine in the selection of a vitamin B_{12}-producer"; S. Yu. Gol'dat (All-Union Institute of Antibiotics) "Selection of Actinomyces aureofaciens (producer of aureomycin-biomycin) by the use of mutagenic factors", V. G. Zhdanov (IAE AN SSSR) "Combined action of chemical and physical factors in selection of an erthromycin-producer", L. I. Erokhina (IAE AN SSSR) "Use of ultraviolet rays in the selection of streptomycin-producing strains", and S. V. Kameneva (IAE AN SSSR) "Selection of penicillin-producers".

These communications were a good supplement to S. I. Alikhanyan's address.

The variation of Actinomyces olivaceus under the action of actinophage was dealt with by T. S. Il'in, A. F. Teteryatnik, I. V. Fedorova, and V. I. Retinskaya (IAE AN SSSR) in the paper "Use of actinophages in the selection of actinomycetes". S. Z. Mindlin, A. V. Vladimirova, and L. N. Borisova (IAE AN SSSR) gave a paper on the theme "Obtention of hybrids of tetracycline-producing actinomycetes (Actinomyces rimosus and A. aureofaciens) and their use in the selection of active strains". It was shown that the antibiotic activity of the hybrid forms of both the investigated species was much greater than that of the parent forms —biochemically deficient variants. A hybrid strain which had considerable advantages over the industrial strain in fermentation on concentrated soya medium was isolated.

In the paper of S. Z. Brotskaya (Institute of Microbiology) "Effect of various doses of UV-rays for the obtention of Aspergillus variants forming active proteases" it was reported that the optimum dose of UV-rays of wavelength 2537 A for the obtention of such variants was 48.0 10^{-3} erg/conidium. A single exposure to ultraviolet did not lead to the appearance of stable strains. A repeat exposure to ultraviolet was required for this.

E. Y. Shcherbakova (VKNII) in a paper entitled "Variation and selection of strains used in industry for obtaining citric acid" gave an account of methods of selecting highly active industrial strains of A. niger.

A special paper of A. A. Imshenetskii was devoted to the coordination of research on microbial selection.

In the discussion of the delivered papers all the speakers noted the timeliness of the convention of the conference and its undoubted value.

A resolution adopted by the conference noted that considerable work on the selection of useful forms of microorganisms had been carried out in the USSR in recent years. However, in view of the tasks set before microbiologists of the USSR by the resolutions of the Twenty-first Congress of the Communist Party of the Soviet Union, the scale of research on the experimental

obtention of useful forms of microorganisms was obviously inadequate. The conference accepted a number of recommendations, the essence of the main ones being as follows. It is essential to expand existing or create new laboratories on genetics and selection of microorganisms in the institutes of the Academy of Sciences, USSR and the Union Republics, and to set up selection groups in branch institutes and laboratories. The proved methods of obtaining active forms—directed rearing (adaptation), the action of radiations and other mutagenic factors, continuous improving selection from industry, and isolation from nature—must be perfected.

Methods of sexual and vegetative hybridization of microbes, transduction, lysogenic conversions, and transformation must be developed.

There must be an expansion and intensification of theoretical research on physiology and biochemistry, on the role and importance of particular structures in microorganisms and viruses in variation and heredity.

For improving the coordination of research the setting up of a special commission under the Scientific Council for the problem "Regulation of Microbial Metabolism" is considered advisable, and this commission would be responsible for coordination of research on genetics and selection of microorganisms.

CONFERENCE ON THE GEOLOGICAL ACTIVITY OF MICROORGANISMS

M. V. Ivanov

Translated from Mikrobiologiya, Vol. 29, No. 3, pp. 467-468, May-June, 1960

An All-Union Conference on the Geological Activity of Microorganisms was held in the Institute of Microbiology of the Academy of Sciences, USSR in Moscow on April 21–23, 1959. The participants in the work of the conference consisted of more than 150 persons —workers in the biological and geological institutes of the Academy of Sciences, USSR, the academies of sciences of the Union Republics, universities, branch geological institutes and other scientific research and industrial organizations.

The aim of the conference was to discuss the present state of research on the role of microorganisms in the geological processes of formation, alteration, and destruction of deposits of certain useful minerals and to outline the main questions for study in the period immediately ahead. Altogether, 23 papers and 12 scheduled addresses were heard.

Great interest and animated discussion was aroused at the conference by the papers of S. I. Kuznetsov "A program of research on the geological activity of microorganisms" and L. D. Shturm "Influence of ecological factors on the microflora of oil deposits", as well as by the long paper of B. V. Perfil'ev on the methods which he had devised for revealing the role of microorganisms in the formation of sedimentary iron and manganese ores in the bottom deposits of waters.

A large group of speakers devoted their addresses to an account of new data obtained in research on the microflora of oil deposits. Special mention should be made of the interesting studies of a group of microbiologists, chemists and geologists headed by K. B. Ashirov (Kuibyshev Scientific Research Institute of Oil Refining) and M. V. Gasanov (Azerbaijan Scientific Research Institute of Oil Extraction), who have demonstrated the exceptionally great importance of sulfate-reducing bacteria in modifying oil waters in processes of accelerated oil extraction by the method of flooding the seam. The data of I. L. Andreevskii, who studied the microflora of selected oil-bearing rocks in the Yarega oil field, were also of great fundamental significance.

An account of the results of numerous investigations on the distribution of microorganisms in underground waters of various regions was given in the review papers of the hydrogeologists M. S. Gur'evich (All-Union Geological Research Institute, Leningrad) and M. E. Al'tovskii (All-Union Scientific Research Institute of Hydrology and Geological Engineering, Moscow), and also in a series of addresses and papers, which included the particularly interesting results of Z. I. Kuznetsova (All-Union Scientific Research Institute of Hydrology and Geological Engineering) who studied the variation in the numbers of bacteria involved in sulfur circulation on a transect through an artesian basin from the supply region to the outflow of the underground waters. We should also mention the interesting paper of O. Yu. Volkova (State Balneological Research Institute for Caucasian Mineral Waters), who demonstrated the ability of sulfate-reducing bacteria to use small amounts of natural organic substances occurring in mineral waters and sedimentary rocks.

The conference noted the need for further research on the role of microorganisms in the metamorphosis of underground waters, since the role of some groups of bacteria such as the cellulose and denitrifying bacteria cannot yet be regarded as adequately clarified.

Review papers on investigations of the microflora of sulfur and sulfide deposits were delivered by M. V. Ivanov and N. N. Lyalikova (Institute of Microbiology, Moscow). During the discussion of these papers wishes were expressed for the organization of microbiological research on the enrichment of poor and thrust ores of heavy metals (V. I. Ivanov, Uralmekhanobr Institute) and on the role of sulfur-oxidizing thiobacteria in the genesis of bauxites (G. M. Karavaiko, Institute of Microbiology).

A very lively discussion developed round the paper of G. A. Mogilevskii, who dealt with some results of the application of bacterial methods of prospecting for oil and gas deposits. The conference noted the need for further improvement of this method and, in particular, for a thorough study of the physiology of the bacteria oxidizing gaseous hydrocarbons.

In addition to scientific problems the conference also discussed several questions of method and administration: the training of specialized microbiologists, the publication of textbooks on methods of studying the geological activity of microorganisms. It was decided that current coordination work should be conducted through the agency of the All-Union Microbiological Society. Several of the scientists who gave addresses (A. A. Imshenetskii, L. M. Rubenchik, A. S. Razumov, R. F. Gekker, and others) noted the great importance of the convention of the Conference on the Geological Activity of Microorganisms, the first of its kind in the history of microbiology in our country.

DEFENSE OF DISSERTATIONS

Yu. N. Karasevich

Translated from Mikrobiologiya, Vol. 29, No. 3, pp. 468–471, May-June, 1960

I

Two dissertations were defended at the Institute of Microbiology AN SSSR on the 30th of December, 1959.

S. Z. Mindlina defended her thesis as a candidate for doctorate in the biological sciences on "The Variability and Selection of Actinomyces rimosus, which produces Oxytetracycline".

It was established that ultraviolet irradiation of the spores of A. rimosus produces significant colonial variation both by morphological and physiological parameters as well as in their antibiotic activity. The frequency of appearance of three types of variants, including variants with increased activity, depends on the dose of ultraviolet radiation.

As a result of stepwise selection of variants induced by ultraviolet rays a highly active strain is obtained from strain 8229 of A. rimosus which produces oxytetracycline, designated LS-T118. Under conditions of production strain LS-T118 exceeds by twofold the productivity of the formerly employed strain 101 of A. rimosus.

It appeared that the selection of nutrative medium had a great deal to do with the selection of the variant. During the selection of UV variants of strain LS-T118 on corn medium containing high concentrations of inorganic soluble phosphorus, strain LS-T293 was isolated, which was adapted to media with increased content of corn extract.

By means of simultaneous growth of biochemically deficient variants, hybrid forms were obtained. A method was worked out for obtaining hybrids with increased frequency.

It was shown that hybrids of A. rimosus are characterized by extreme variability in their morphological characteristics and antibiotic activity. Several hybrids exceeded the parent strain in their activity.

Thus hybridization can be used together with mutational factors as a means of increasing the natural variability of A. rimosus. The use of hybridization undoubtedly opens new perspectives in the selection of actinomycin-producing strains.

II

Z. I. Yuzhina defended a dissertation as a candidate for a doctorate degree in the biological sciences on the "Role of Microorganisms in the Toxicity of the Soil of the Kolsky Peninsula".

It was established that virgin soils of the Kolsky Peninsula are toxic for azotobacteria. It appeared that, of the genetic lines of virgin soil, the most toxic properties were found in line A_0. Different survival of azotobacteria was seen in lines B and C.

During the cultivation of humus, iron-bearing podzol, humus podzol, marsh land, and meadows, their toxicity is appreciably decreased. The cultivation of iron-bearing podzol and higher and transitional marsh land does not lower their toxicity for azotobacteria.

Many cultivated and virgin soils of the Kolsky Peninsula are toxic for plants. However, plants are not as effective in testing the toxicity of the various types of soils.

It was shown that azotobacteria are a more sensitive indicator for the toxicity of the soil of the Kolsky Peninsula than plants. However, not all soils which are toxic for azotobacteria are toxic for plants. This limits to some extent the use of azotobacteria as an indicator for the determination of toxicity of soil for plants.

The degree of toxicity of both virgin and cultivated soil on the Kolsky Peninsula for azotobacteria depends on the number of microbes in the soil which inhibit azotobacteria.

It was established that the common antagonists to azotobacteria in the soil are molds of the Penicillium genus and spore-forming bacteria of the Bacillus mesentericus group. The correlation between molds and bacteria and the toxicity of the various soils is variable.

It was shown that antagonists of azotobacteria are also toxic for plants. The most active inhibitors were the molds of the Penicillium genus.

The introduction into weakly toxic or nontoxic soils of the most active cultures of azotobacteria and plant antagonists increased the toxicity of the soil.

$CaCO_3$ is an effective means of lowering the toxicity of soil of the Kolsky Peninsula.

A correlation between the toxicity of soil and its pH was not demonstrated. In some instances increased toxicity of the soil resulted in lowered or stationary pH and in others increased toxicity resulted in increase in pH. However, the toxic soil mostly had a pH less than 5.

III

Two dissertations were defended at the Institute of Microbiology AN SSSR on January 27, 1960.

R. A. Zhukova as a candidate for a doctorate degree in the biological sciences defended a thesis on "Cellulose Metabolism of Aerobic Bacteria in Relation to the Toxicity of the Soil of the Kolsky Peninsula".

It was established that aerobic cellulose bacteria are widely but unevenly distributed in the virgin soils of the Kolsky Peninsula. The smallest numbers of them are found in the mountain-tundra soil, the sandy, iron-bearing podzol and in upper marsh land (from 10 to 1000), and the largest numbers in the low-lying marsh land (from 10 to 1,000,000). In the virgin soils as well as in the peat bogs of the upper and transitional marsh land the splitting of cellulose is brought about chiefly by molds and actinomycetes.

It appeared that the number of aerobic cellulose bacteria increased with cultivation of podzol and peat bogs. Liming the soil, manuring, fertilization, nitrogen, and phosphorus introduction as well as the seeding of leguminous grasses particularly favor the development and activity of cellulose bacteria.

In virgin and cultivated soil the number of aerobic and anaerobic cellulose bacteria is not great in comparison to the general microbial population and the presence of ammonia- and nitrogen-fixing bacteria. Aerobic cellulose bacteria in virgin podzol form ten per cent of all the microorganisms. The splitting of cellulose in soil is at best a slow process.

It was shown that myxobacteria are more widespread in the soil of the Kolsky Peninsula than vibrio. Pure cultures of myxobacteria and vibrio were obtained from the soil.

It was established that the cellulose-splitting activity in podzol and peat bog soil proceeds most actively at a temperature from 20 to 27°. At temperatures approaching zero (from −2 to + 4°) the intensity of the splitting process is decreased four to five times.

The optimal temperature for the growth of pure cultures of Sporangium cellulosum and Sporangium cytophaga myxococcoides is around 24 to 28°, and 15 to 28° for Vibrio vulgaris.

Aerobic cellulose bacteria grow in wide pH ranges. Acidity suppresses their viability. The pH optimum for the splitting of cellulose by S. myxococcoides is 7.3 to 8.1, by S. cellulosum is 7.9 to 8.6 and by V. vulgaris is 8.2 to 8.6.

The majority of the soil in the Kolsky Peninsula is toxic for aerobic cellulose bacteria. The degree of toxicity varies with different soils. The greatest suppressive effect on the growth of cellulose bacteria is achieved by podzol, and podzol marsh land and soil in the upper and transitional marshes. The primitive, poorly developed lands of the meadows and lower marshes only insignificantly suppress growth. Cultivated soil is as a rule less toxic than virgin soil.

Correlation between the pH of the soil and its toxicity for aerobic cellulose bacteria was not observed. However, acid soil was largely very toxic.

The soil of the Kolsky Peninsula is exceptionally rich in microorganisms capable of inhibiting or completely suppressing the growth of aerobic cellulose bacteria. In unfertile iron-bearing podzol and in upper and transitional marsh land the largest number of antagonists to cellulose bacteria is found in those soils which are very toxic for these bacteria. The author presumes that cultures with definite antagonistic properties play a significant role in the toxicity of the soil.

The toxicity of the soil is not singly due to different forms of aerobic cellulose bacteria.

Spore-forming bacteria of the Bac. mesentericus group and molds are the basic antagonists to cellulose bacteria in the soil of the Kolsky Peninsula. From 39 to 56% of the molds in cultivated and virgin soils are antagonists to cellulose bacteria. In the very toxic soil the content of antagonistic molds is confined to representatives of the Penicillium and Trichoderma genera.

The introduction into nontoxic or weakly toxic podsol soil of the strongest antagonists to cellulose bacteria evokes an increase in the toxicity of the soil for these bacteria.

The limited distribution and activity of the aerobic cellulose bacteria in the virgin and cultivated soil of the Kolsky Peninsula is dependent not only on the unfavorable physicochemical properties of the soil (low temperatures, acidity, and insufficiency of nitrogen and phosphorus) but also to a significant degree on biological factors such as the presence of large numbers of microbes which are antagonists to cellulose bacteria.

To increase the splitting of cellulose the author recommends that in cultivation of the soil of the Kolsky Peninsula these agricultural techniques be applied to decrease the toxicity of the soil for aerobic cellulose bacteria. In this regard he refers to liming, manuring and the seeding of cereal, leguminous grasses.

IV

T. S. Ilyina defended a thesis submitted for a doctorate in the biological sciences on "The Variability Induced by Actinophage in Actinomyces".

A high frequency of morphological mutations were experimentally established with actinophage, attaining in some instances an incidence of 98%. It appeared that the effect of actinophage on actinomyces cells had a specific character both in relation to frequency and the character of the mutation. On the basis of these data the author hypothesized that phage-resistant variants were not present in the original culture but arose directly as a result of the actinophage effect.

Biochemically inadequate mutants occurred as a result of actinophage activity. The frequency of biochemical mutation was 2.8 times greater than that induced by x-rays.

The variability of strain H-6 Actinomyces olivaceus after treatment with actinophage No. 1 resulted in both active and inactive variants. At the same time treatment of this strain with actinophage No. 2671 resulted in the formation of more inactive than active variants. Treatment of actinophage with DNAase did not inactivate the mutagenic character of the actinophage. Inactivation of phage fractions by heating completely destroyed the mutagenic effect. From this it would appear that the variability induced by the phage resides within the phage fraction.

An effect of the original culture on the phage was also demonstrated. It appeared that actinophage grown

in different types of actinomyces brought about morphologic variations of different kinds. It was shown that the actinophage transmitted characteristics from the donor to the recipient cells. Experimentally obtained lysogenic variants demonstrated that lysogeny is of significance in the variability of strain H-6 A. olivaceus.

The experimental data presented pointed out the significant role of actinophage in the variability and evolution of actinomyces.

PROFESSOR N. A. NAUMOV (ON THE ANNIVERSARY OF HIS DEATH)

A. A. Babayan

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Professor Nicholas Alexandrovich Naumov, corresponding member of the Academy of Sciences USSR since 1946, doctor of agricultural and biological sciences, died on the 6th of July, 1959, in the 72nd year of his life, that of an outstanding plant pathologist and mycologist of our country.

N. A. Naumov belonged to that constellation of Russian scholars, who, at the threshhold of the October revolution, already had renowned scientific achievements. Nonetheless his investigative and pedagological talent came to its greatest fruition during the Soviet era.

Being one of the first students of the outstanding plant pathologist and mycologist A. A. Yachevsky, N. A. Naumov, thanks to his talent, diligence, and great knowledge, rapidly rose to the front ranks. Never losing his connection with the laboratories of the All-Union Institute for Plant Conservation, both while A. A. Yachevsky was alive as well as after his death, N. A. Naumov himself founded several scientific centers for the study of mycology and plant pathology, among which are those at the Leningrad University and the Agricultural Institute where he headed the department of mycology and plant pathology.

N. A. Naumov was equally interested in mycology and plant pathology and he possessed great erudition in both these fields of science. In our time, the successful solution of agricultural and biological problems requires greater specialization. This is a logical consequence of the successful development of scientific thinking in our country on the basis of the growth of numerous departments in different scientific branches. But in the early period of his activity a broader spectrum of knowledge was demanded of N. A. Naumov, who was a highly talented and tirelessly diligent man, and mastered this task and acquired great experience in the areas of mycology, phytopathology, taxonomy, plant physiology, physics, chemistry, plant toxicology, microtechnology, the mycotic diseases of man and animals, and so on. Due to this, various organizations and private individuals turned to him yearly for consultation with a great variety of problems and Nicholas Alexandrovich never left one of these problems without an answer.

Of the most important investigations of N. A. Naumov one need only cite the studies on the phenomena of cereal rusts, plant viral diseases and non-parasitic diseases, on fungicides and the problems of plant immunity and so on. His monograph on cereal rusts is an example of concise, conscientious and critical exposition of a vast, world-wide literature and serves as a reference for individuals interested in biology and measures for combatting this unique group of parasitic fungi.

N. A. Naumov gave his greatest service to his country's agriculture in preparing groups of agronomists, plant pathologists, mycologists, and scientific workers in these fields. A course composed by him on ailments of agricultural plants has had several editions and is still used in all the institutions of higher learning in our country. He also had published several useful books on microscopic technique in plant pathology (1932), on methodology in mycology and plant pathology (1937), and on the fundamentals of botanical microtechniques (1956, coauthored with V. E. Kozlov). These works were mainly based on his lectures and undertakings which he carried out at the Leningrad Institute of Applied Zoology and Plant Pathology from 1922 to 1928.

N. A. Naumov edited the posthumous edition of the voluminous works of A. A. Yachevsky "The Fundamentals of Mycology" and "Plant Bacteriology" and also a number of reference books on agricultural plant ailments, to which a number of specialists contributed.

N. A. Naumov devoted much energy and attention to the study of the mycological flora of the country and the development of this branch of science in the Soviet Union. Representative molds of the Leningrad region, the Urals, Crimea, and other parts of the country were studied in great detail by him. No less than 160 new species and 20 new genera were described by him.

Of his papers on mycology during the first period the monograph on wheat molds deserves special attention. This work was published both in the Soviet Union and in France where it appeared in 1939 as the ninth volume of the Encyclopedia of Mycology.

In the preface to the edition of this highly prized work, the president of the Botanical Society of France, Prof. Pierre Allorge, wrote that the work of Prof. Naumov "can be viewed as an example of clarity and the power of scientific erudition, based on many years' experience and the faculty of a critical approach".

From 1920 N. A. Naumov actively participated in the work of the Section of Mycology and Plant Pathology of the All-Union Botanical Society, of which he was at first secretary then chairman pro tem, and finally chairman. The accuracy and conscientiousness of Naumov characterized the sessions of the Section, convened by him in the years of his secretariat (1920–1932), the majority of which was published in "The Materials of Mycology and Plant Pathology" (1922–1931) and has great interest for the many facets of the

historical development of this branch of science in our country.

N. A. Naumov had many students attending his lectures and acquiring under his direction the habits of investigative and pedagogic work. Exceptional modesty and simplicity in dealing with students and subordinates won great respect for N. A. Naumov and his students and coworkers always generously took advantage of his knowledge and experience.

At the present time many students of N. A. Naumov in different regions and republics of our multinational country have leading positions in the areas of plant pathology, mycology, and plant conservation.

N. A. Naumov enjoyed popularity and authority not only in our country but also abroad. Back in 1928, before the creation of the international plant pathology journal, "Phytopathologische Zeitschrift," published in Berlin, he was included in the first board of editors by his colleagues. He was a member of the Mycological Society of France, the Lyons Society, the Society of Applied Botanists of Germany. He was also the member of many permanent commissions on taxonomic problems in mycology for the international botanical congresses.

For his investigations and as a public figure N. A. Naumov was awarded orders and medals of the Soviet Union by the government.

N. A. Naumov has spent his earthly life sown with work and creation. A tireless worker, great scholar, outstanding citizen, and modest man—these are the qualities which characterize the personality of Nicholas Alexandrovich Naumov.

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya.	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

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